insects

Insecticides for Mosquito Control

Strengthening the Evidence Base

Edited by

Rosemary S. Lees

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Insecticides for Mosquito Control: Strengthening the Evidence Base

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Editor

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Cover image courtesy of Rosemary S. Lees.

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About the Editor

Rosemary S. Lees

Rosemary Lees (Dr) leads the Methods Validation workstream of I2I. She is working with the Technical Team to identify areas of methodological weakness in the development and evaluation of vector control tools and seek to address these gaps through the development of consensus protocols, experimental validation of new and existing methods, and discussion with stakeholders to map out key areas of concern and generate consensus on solutions. She is a Senior Technical Officer in the Vector Biology Department of LSTM, working with Professor Hilary Ranson on research into insecticide resistance mechanisms and their impacts on vector control, and with industry partners to screen and evaluate the potential of new insecticides, formulations and products as well as application equipment and related technologies.

Preface to "Insecticides for Mosquito Control: Strengthening the Evidence Base"

The control of diseases spread by mosquitoes relies heavily on the tools used to control these vectors, which are commonly based on chemical insecticides. In the face of increasing insecticide resistance and other challenges it is critical that the toolbox of insecticides and tools continues to grow, and decisions about how to spend limited public health resources must be made using robust evidence about their efficacy.

This book presents original research into developing and characterizing new vector control tools, as well as understanding and monitoring insecticide resistance. Review articles explore the impact of insecticide resistance and offer guidance on insecticide choice in the face of pyrethroid resistance. Consensus methodologies are presented, in the form of standard operating procedures (SOPs) designed to be adopted and used to generate reproducible data that can be compared and interpreted across and between studies.

It is hoped that this collection of valuable articles will offer inspiration and guidance to researchers and those developing, procuring and deploying vector control tools on how consistent data can be generated and used to inform more effective development, evaluation, and use of new and existing vector control tools.

The cover image is a photo of petri dishes containing filter papers attached to a wall with water sensitive papers in a grid arrangement I took during a study to map the spray distribution applied by a vertical track sprayer (see page 55).

Rosemary S. Lees
Editor

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Editorial

Insecticides for Mosquito Control: Improving and Validating Methods to Strengthen the Evidence Base

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1. Background: Good Decisions Require Good Data

Efforts to eliminate vector-borne diseases, for example malaria which caused an estimated 619,000 deaths in 2021 [1] or arboviral diseases such as dengue and zika [2], rely heavily on the use of vector control tools. The toolbox available to combat insect vectors of disease is growing through improvements to existing approaches and new, emerging, technologies. New chemistries are being developed to target pyrethroid-resistant malaria vectors, for use in conventional tools such as insecticide-treated nets (ITNs) and indoor residual sprays (IRS), as well as through innovative means of deployment such as attractive targeted sugar baits (ATSB), passive emanators and eave tubes. Rear and release strategies to control *Aedes* vectors of arboviruses are under pilot evaluation, including versions of the sterile insect technique (SIT) and the use of *Wolbachia* symbionts for population control or replacement. These tools are also being piloted to urgently combat the expansion of *Anopheles stephensi* in Africa.

The decision to deploy new vector control tools or approaches on an operational level should be supported by robust entomological evidence to demonstrate efficacy, comprising data collected using appropriate and validated methods. A strong evidence base can also guide effective operational deployment decisions. The Insects Special Issue "Insecticides for Mosquito Control: Strengthening the Evidence Base" presents original research into developing and characterising new vector control products, as well as understanding and monitoring insecticide resistance. Review articles explore the impact of insecticide resistance and offer guidance on insecticide choice in the face of pyrethroid resistance. Consensus methodologies are presented, in the form of standard operating procedures (SOPs) designed to be adopted and used to generate reproducible data that can be compared and interpreted across and between studies. It is hoped that this Special Issue offers inspiration and guidance on how consistent data can be generated to inform more effective development, evaluation and use of new and existing vector control tools.

2. The Impetus to Better Validate Entomological Methods

Issues around improved generation and interpretation of entomological data are particularly timely in light of the establishment in 2016 and ongoing evolution of the World Health Organization (WHO) Prequalification Vector Control Team (PQ/VCT), whose mandate is to "increase access to safe, high-quality and effective VCPs" (vector control products) by working to evaluate and prequalify tools and contribute to building assessment capacity in national regulatory authorities. Prequalification streamlines access to vector control products by employing regulatory best practices to product evaluation and provides relevant information to help guide decisions about procurement and implementation. However, robust decisions can only be made using high quality, consistent data.

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Implementation of good laboratory practice (GLP) compliance in an international network of vector control testing facilities was an important first step to improving quality of entomological data [3]. GLP compliance offers reliable, auditable, and reproducible data, but does not guide on testing methodology. Standard protocols are available in WHO guidance and elsewhere for evaluating the bioefficacy of vector control tools, but these were developed primarily to measure the fast-acting lethal effect of pyrethroids in contact assays and may not be appropriate for new modes of action or methods of active ingredient (AI) delivery. In order to spur innovation, it is crucial that validated methods are available for tools with different modes of action to measure the relevant end points with sufficient accuracy, sensitivity and reproducibility.

This Editorial will consider in detail the issues relating to entomological efficacy testing of insecticides and insecticide-based products targeting adult *Anopheles* mosquitoes, with a particular focus on ITNs and IRS. However, the illustrations of good practice and recommendations highlighted here are more widely applicable to other types of tools, as evidenced by many of the articles included in the Special Issue [4–6]. This includes tools targeting *Aedes* mosquitoes [7] where the commercial market is consumer-driven, the regulatory framework is more flexible and the guidance on evaluation sparser. There are gaps in the guidance for the evaluation of larvicides and spatial repellents, for both *Aedes* and *Anopheles* control. Many issues highlighted in this discussion will also be relevant for the collection and interpretation of epidemiological data relating to the efficacy of vector control tools, and to the need to better characterise the mode of action and impact of new tools [8,9].

The most widely used methods for measuring the insecticidal bioefficacy (i.e., the ability of the insecticide component(s) of a vector control tool to kill susceptible target vectors) of malaria vector control tools include cone bioassays, tunnel tests, and experimental hut trials (EHTs) [10]. Cone bioassays have been used successfully to evaluate and monitor ITN and IRS bioefficacy for more than 20 years and have proven able to generate GLP-quality data for two important, rapidly induced, easy to measure outcomes: mosquito knockdown and mortality. These outcomes, which are usually measured around 60 min after exposure for knockdown and 24 h after exposure for mortality, are critically important factors that influence how effective a vector control tool can be at preventing disease transmission and are, therefore, important to monitor over time. However, these knockdown and mortality endpoints, similar to the cone bioassay initially implemented to measure them, were designed specifically to easily test the immediate effects of fast-acting, topically exposed neurotoxins such as DDT and pyrethroids. They were never intended, or adequate, for capturing the full range of insecticidal modes of action on vector biology or disease transmission.

These shortcomings led to the inclusion of additional outcomes that are typically measured with tunnel tests and EHTs, and measures such as blood-feeding inhibition, deterrence, induced exophily, and delayed mortality that have become more widely used [11]. Tunnel tests and EHTs have proven useful for monitoring the efficacy, and predicting the effectiveness of, vector control products over time and for guiding product development. However, their outcome measures have proven more difficult to standardize, replicate, and interpret across a diverse range of research and implementation settings. These difficulties are being exacerbated by the arrival to market of new active ingredients (AIs) with novel modes of action–for example, the delayed mortality induced conditionally by chlorfenapyr [11] and others and the reduced mosquito fecundity induced by pyriproxyfen [12]. Additionally, complicating the testing landscape are new tools that combine insecticides from different insecticidal classes with different modes of action, whose bioefficacy needs to be evaluated independently using multiple mosquito strains and/or endpoints [13], and novel interventions that utilize alternative routes of insecticide delivery distinct from the tarsal exposure to ITNs and IRS, for example the ingestion of insecticidal AIs facilitated by ATSBs [4].

3. The Need for New and Improved Methods

If we are to properly understand the entomological effects of new products and evaluate them robustly, we need to use well-validated methods. Using methods which are fit for purpose is crucial to enable regulators, procurers and implementers to assess and compare the range of tools available to them in order to make informed decisions on how best to utilise limited operational budgets. Clearly, innovation in testing methodology is needed so that testing outcomes describe all the important aspects of a product's insecticidal efficacy but avoid collecting extraneous information or data that is ultimately tangential to distinct product claims. Without such methods, it is difficult to appropriately monitor the entomological effects of a new tool deployed at scale. This becomes especially important in situations where the introduction of a new vector control tool does not have the expected epidemiological impact–without measuring the appropriate entomological efficacy endpoints using the best available methods it will not be possible to elucidate why this might be. For new products in development, it is important to understand not only *that* a product class has an impact, but also *how* it achieves this impact.

Standardised and/or characterised inputs will help to reduce methodological error and variability in the data and help with interpretation of results. One key input is the insects used for testing. The insecticide resistance profiles of the various mosquito strains used for testing should be characterised, be they 'susceptible' or 'resistant' strains [14–16]. The cohorts of mosquitoes used for testing should be generated using standardised rearing, and fitness parameters such as size recorded [14]. In addition to the insects, it is important that the key testing parameters should be standardised or characterised as far as possible, for example using tools such as the Micron Track Sprayer to improve consistency of insecticide application [17], by interrogating methods to identify and minimize possible sources of variability [18], and by optimising methods to improve the consistency of the data [19]. Standardised methodologies such as SOPs generated by consensus [13,14] will facilitate a comparison and interpretation of the results between testing sites and across studies. Data collection should be made as objective as possible, for example by the use of automated scoring tools [20]. It is also best practice, when reporting results of a study, to include methodological detail alongside the data and ensure that raw disaggregated data, including control data, is presented.

We rely on data from efficacy and insecticide resistance testing to make choices about product use and understand the cross-resistance risk for different AIs. However, even with pyrethroids, there is ambiguity in the data generated by accepted methods and a need to understand the sources of variability and characterise or standardise inputs. For example, there is insufficient evidence from testing data or our understanding of the mode of action to confidently recommend the rotation of different pyrethroids for resistance management [21].

The establishment of the WHO PQ/VCT has changed the way in which products are evaluated by WHO, bringing these processes more in line with best practice regulatory approaches and offering a significant opportunity for the robust evaluation of new product classes. PQ/VCT's approach is flexible, allowing applicants to agree methods with WHO that best reflect their product's mode of action, rather than adhering to rigid, standard methodologies. This process still requires any data generated to be robust, consistent and appropriate to the product and its specific mode of action and claims. It is a significant step forward to support innovation in vector control; however, the approach has highlighted long standing issues in the generation and interpretation of entomological data to evaluate new tools. Investment in validating methods to generate robust entomological data has been lacking, meaning that potentially effective tools are unable to smoothly progress through regulatory processes or be consistently evaluated in the field. Developing and validating methods that are fit for purpose will help to streamline decision-making processes by better defining the effect of a tool and reducing the need for the generation of additional supporting data.

Beyond introduction to the market, there is a need to manage the lifecycle of a product, for example determining whether an ITN is performing as expected throughout its 3-year lifespan. If effective methods are not available to measure a product's performance during its active life, it is difficult to measure the appropriateness for implementation in a given context. Entomological data are important for monitoring durability of ITNs, but it should be supported by chemical analysis of the total AI content, or ideally the surface content and presentation of AI [22]. Improvements and adaptations to new product types are also important for analytical methods [23].

4. Relating Results of Laboratory and Semi-Field Tests to Product Performance

It is important that there is clarity on what entomological endpoints should be measured in laboratory, semi-field and field experiments, in order to inform our understanding of how a vector control product will perform. Taking ITNs as an example, efficacy is described in the new WHO Guideline for the Prequalification Assessment of Insecticide-Treated Nets [22] as being influenced by potency, biologically available fraction of the surface concentration of AI, net construction, uptake of AI by free-flying target vectors, as well as handling and care of the ITN. Bioassays in the laboratory can be used to ascertain the efficacy of a product against lab strains and to some extent wild populations under controlled conditions, giving a measure of surface available insecticide across a net and its potency through uptake of this fraction. Semi-field bioassays can provide additional measures of uptake of insecticide by free-flying mosquitoes under more 'real life' conditions. These parameters predict effectiveness, or how well the net may perform in the real world in terms of entomological and, potentially, epidemiological outcomes [24].

Such sequential testing using increasingly sophisticated methods has been the accepted approach to determining efficacy and predicting effectiveness. The new WHO PQ/VCT approach, however, allows more flexibility, facilitating, for example, the progression of slower-acting toxicants and pro-insecticides such as chlorfenapyr. Standard lab bioassays were developed to measure the effect of fast-acting pyrethroids, and measure endpoints which are not appropriate for a pro-insecticide. Unlike pyrethroids that kill by acute neurotoxicity, chlorfenapyr kills by disrupting a mosquito's ability to produce energy in the mitochondria, after it has first been metabolised into its active forms. The more physiologically active a mosquito, the greater the likelihood of higher conversion rates to these active forms. This process of conversion is also enhanced by biochemical processes such as metabolic activity of P450 enzymes. Thus the metabolic state of the mosquito is extremely important during testing. As a result, chlorfenapyr-treated ITNs perform poorly in the lab under artificially controlled testing conditions using standard methods [25], but better in semi-field testing [26,27], and have been shown to have a significant epidemiological impact [28]. This example illustrates the need to use appropriate and validated methods to evaluate a given product or product type, and to avoid the over-interpretation of entomological data.

The results of bioassays and semi-field studies should be interpreted with caution as a prediction of performance of an ITN, defined by WHO PQ/VCT as its ability to provide continuous controlled release of insecticide and maintain physical integrity under normal use [22]. The link between bioassay data and entomological or epidemiological impact when a product is deployed is even less clear, and results should not be conflated to make implementation decisions. To take the ITN example, durability monitoring should measure the effective life of an ITN whereby bioassays are a proxy for surface AI availability and should not be conflated with effectiveness. Laboratory washing methods are used to measure regeneration time and as a means to artificially age nets, but they may not reflect the treatment of nets under normal use conditions [29]. In IRS testing, the walls of experimental huts treated with test products to measure residual efficacy may not accurately represent the results of real-world application, though new methods and equipment can at least be used to improve accuracy of wall treatment [17]. Methods should be chosen or designed to accurately measure the intended entomological endpoint for a given purpose,

and we must be consistent about how the data are interpreted and careful not to conflate results from testing carried out for different purposes or measuring different endpoints.

Monitoring of insecticide resistance in target vector populations is another example of bioassay data being used beyond the scope of questions methods were designed to answer. Using a discriminating concentration to test mortality in wild caught mosquitoes should be routine practice to monitor for emergence of resistance in a vector population as a warning sign that a product may start to fail [30]. The WHO cylinder and bottle tests were designed to provide information about intrinsic susceptibility to the insecticide, not as a predictor of product efficacy, or to predict product effectiveness at a given location. Further, susceptibility testing methods may need to be adapted to consider different means of deploying insecticides, for example ingested insecticide in attractive targeted sugar bait (ATSB) products [4].

We are left with the question of how far we might be able to link entomological end points, measured through bioassays, and product impact. Better understanding of existing testing methods and how to use the data they generate will be key. For example, EHTs start to bridge the gap between cone tests and entomological impact and are the gold standard, but the link between EHT results and resistance is highly uncertain [31]. Recent analysis shows that EHT data can be used to parameterise models and reliably predict epidemiological impact of rapid-acting pyrethroids on ITNs and IRS [24]. Conversely, modelling may be used to more meaningfully interpret and use the data generated by laboratory and semi-field bioassays. Additional data may be generated while applying existing methods, for example by measuring not just knock down or mortality after exposure to an insecticide-treated product but also measuring sublethal effects of exposure [8]. Delayed mortality, reduced fecundity, reduced blood feeding and other parameters may result in entomological impact. It is important to observe end points that are relevant to the mode of action and intended effect of the product under evaluation, which relies on understanding the wholistic impact of insecticide exposure on mosquito populations [9]. Sublethal effects of insecticide exposure are much more important to understand in the case of highly resistant populations, and slow-acting mortality is important to measure and understand for different modes of action.

If we were able to more directly connect the results of small-scale entomological experiments, enhanced by using a range of well-characterised vector colonies to reflect a wide range of target populations [16], to entomological and ultimately epidemiological impact, then we could potentially reduce the need for costly and lengthy clinical trials and speed the route marketing new products. This has been one aim of the New Nets Project, which is implemented by a consortium of partners led by the Innovative Vector Control Consortium (IVCC) to build the evidence needed to influence policy in this area [32] through enhanced data collection during randomised control trials and under operational pilot conditions. In some cases, it may be necessary to develop new methods to collect the evidence that is needed. An example is provided by the Ifakara Ambient Chamber Test (I-ACT) [33], which allows more controlled and high throughput evaluation of the efficacy of vector control products in semi-field conditions and provides greater statistical power than an EHT; thus, it is an important additional method.

5. The Need for Pre-Emptive Method Development and Validation

For vector control products with novel modes of action that are considered "first in class," the WHO Vector Control Advisory Group (VCAG) requires two epidemiological trials to demonstrate public health value before a product class can be recommended and a "first in class" product can receive a WHO prequalification listing. Any subsequent product that elicits a similar entomological effect should be able to receive a policy recommendation based on accepted and validated entomological methods with well understood links to epidemiological outcomes. This has been demonstrated for pyrethroid-only nets, where EHTs predict performance well [24], and a similar analysis is underway to correlate hut trial results for chlorfenapyr-containing nets with field performance. However, when

considering lab-based assays, any correlation for chlorfenapyr is challenging because commonly used methods do not capture its mode of action effectively. When undertaking efficacy testing for novel vector control chemistries/products, it is important to understand not just what the entomological effect is, but also how that effect is produced.

By using appropriate methods, that are validated before a product is brought to market, bottlenecks to access are reduced and new tools can be adopted with higher confidence in their performance. Additionally, there may be scope to improve products after launch if we understood them better, for example by selecting a more active crystalline form of an insecticide to use in IRS [34]. A good example of a testing pipeline for a novel insecticide from mode of action to method of deployment is described by Mysore et al. [5]. Innovative new approaches need clear guidance on the data required to demonstrate efficacy and the methods to collect them. Plant-based compounds [6] or RNAi approaches [5], for example, may be used to circumvent resistance, but it's important to ensure that WHO guidelines for efficacy and resistance testing are appropriate for these alternative AIs and tools. Similarly, efficacy testing of products developed without insecticides such as bite-resistant fabrics [7] are not covered by current guidelines or standard methodologies.

There is a need for a broad and robust data set before a new product is brought to market, which relies on having robust methods to measure entomological endpoints, as well as solid interpretation, analysis and use of the data generated. WHO PQ/VCT would ideally be evaluating products using data generated with validated methods, but thus far no guidance is available as to what method validation should consist of, beyond the Collaborative International Pesticide Analytical Council (CIPAC) methodologies which focus on analytical chemistry [35]. CIPAC presents a clear framework for validation; however, it is a challenge to apply analytical standards to bioassays as they are not realistic for a biological system. There is a need for rigorous standards and validation of methods, but until recently there has no guidance on how to perform validation of entomological methods [36]. This gap has resulted in products going into use before there are established methods for durability or resistance monitoring, whereas is it important that sufficient baseline data is collected to monitor for loss of efficacy with use or reduced susceptibility as an insecticide-based product is deployed [4]. For some products this could mean that there is already unidentified cross-resistance in the target population. In some cases, the only methods available for their evaluation can only be performed in certain sites, for example the tunnel test currently relies on access to an animal host to perform well [19]. In other instances, it will be important to understand possible interactions between products used in integrated vector management, and those methods also need to be developed [37].

6. Conclusions: A Timely Opportunity to Drive Improvement in Method Validation and Evidence Generation

The establishment of the WHO PQ/VCT process, and the welcome focus on a regulatory approach to product evaluation, offers the opportunity for a fundamental change in the way we view vector control products. The promise of this new process is that product developers can generate data that reflects the performance of their product, rather than developing products to meet a rigid set of data requirements. There is an opportunity to move away from thresholds applied to bioassay results and used to judge efficacy of products, and towards a more rounded "weight of evidence" evaluation which allows a greater understanding of how a specific product works, making it easier to compare it to other classes and meaningfully monitor ongoing efficiency. This in turn should help to correlate entomological endpoints with product efficacy and impact, and will allow more informed procurement and deployment decisions. However, this flexibility must be underpinned by robust, reproducible data that clearly support product claims that are independently validated. The fact that many current entomological methods have been designed to measure the rapid kill and knockdown of pyrethroids hinders the exploration of different modes of action or methods of delivering AIs. As such, investing in methodological development is key to help spur future innovation in vector control.

Validation of methods is crucial to ensure that results are reproducible and informative. Even seemingly standard methods, such as the WHO tube test, show significant variation if testing is not standardised [18]. Innovation can be applied to improve data generation even with standard methods [19]. Taking steps to utilise methods that accurately detect relevant entomological endpoints will be crucial to interpreting and comparing new tools with different modes of action. This will be complicated by the wider range of entomological effects induced by new AIs, and the relative impact of the same level of, for example, sterilisation or delayed action mortality may not be obvious. Data should be generated using validated methods that are characterised in terms of the natural variability of data and more effort must be made to characterise material inputs to provide further context to results. The responsibility for method development and validation for product evaluation and monitoring lies primarily with manufacturers, who understand their products best, with the support from the research community on innovation and development. There is a potential role for an independent body tasked with the validation of proposed entomological methods on behalf of the manufacturers, analogous to the role of CIPAC for methods in analytical chemistry.

Currently, data generation for vector control tools is centered around access to the market with a focus on a WHO policy recommendation and WHO PQ/VCT listing. These are important milestones, but data is needed throughout a product's lifecycle to inform on performance and aid deployment decisions, particularly when implementing resistance management strategies. Recent history has seen the development of lifecycle management methods only occurring post market, meaning products in use may not have reliable methods to generate data on performance trends and ascertain resistance issues when first launched. We therefore recommend that a comprehensive package of data is generated for a vector control product that goes beyond market access and encompasses lifecycle management. This should include a package of validated methods for generation of the data throughout a product's lifecycle, and a means for interpretation to assist decision making for implementation. Some of this information will be available through established evaluations (e.g., WHO PQ/VCT), but others should be considered in addition to those requirements to ensure streamlined uptake of new tools.

- **Scope of the product:** detailed description of, for example, under what conditions it is expected to be effective, the target species, and what resistance mechanisms exist in the target population/s which might be relevant.
- **Entomological mode of action**: as detailed a description as possible about how the product acts on the target species to elicit the intended effect, which may include IRAC classification [38] and is important to the understanding of cross-resistance risks and potential interaction between products.
- **Intended entomological endpoints:** clear definitions of the entomological effects which are relevant to the product and should be measured to demonstrate efficacy.
- **Regeneration time:** clear understanding of the dynamic presence of insecticide within the product or its sphere of influence, for example the time taken for AI to regenerate on the surface of an ITN after washing.
- **Insecticide content and formulation:**
    - The functionality of an insecticide-based product depends on the amount of insecticide present (a) in total, (b) on the surface, and (c) in the bioavailable fraction which vectors are able to pick up. Insecticide content needs to be monitored throughout the life cycle of a product.
    - Knowing the way that a product is formulated and the insecticide is presented to the mosquito is also critical to understanding how a product works, for example whether an ITN is coated with an AI or the AI is incorporated into the fabric, or whether an IRS formulation is a suspension or microencapsulated formulation. The presentation of AI may change over the life of the product.
    - An understanding of both insecticide content and presentation is needed in two settings:

1. Under standard conditions in the laboratory, where, for example, it may be sufficient to use analytical methods to measure AI concentration since the aim is to understand the properties of the product and monitor quality.
2. Under real world conditions, where the aim is to understand a product's effectiveness and so bioassays should be used in place of, or to confirm, analytical methods.

- **Residual efficacy**: three elements of a product should be monitored over time:
  - **Bioefficacy**, or the ability of the product to elicit the intended entomological effect. This may be measured through a bioassay as a proxy measure for the bioavailable fraction of AI, or an analytical method which has been shown to correlate with the results of a bioassay.
  - **Physical durability**, a measure of the ability of the product to resist physical damage or degradation under real-world use. May be measured through monitoring products post-deployment or by artificially recreating the conditions of real-world use, for example the use of standardised washing methods to mimic the use of ITNs.
  - **Resistance monitoring**, which should include a defined discriminating dose and method of exposure to monitor for the decreased sensitivity of the target species to the AI/s.
- **Interaction with existing tools or AIs:** vector control tools do not exist in isolation and multiple tools which may be deployed in an integrated manner or inadvertently be used together in the same location.
  - It is important to understand how the effectiveness of a new product may affect or be affected by other tools which may be used in the same location, for example an emanator with a repellent effect may reduce interaction of mosquitoes with an ITN and reduce its killing effect, or a synergist on an ITN may reduce the lethal effect of a pro-insecticide in an IRS formulation.
  - The cross-resistance risk needs to be considered for a new AI deployed in an area where resistance mechanisms already exist in the vector population as a result of exposure to other AIs.

Although the focus of this Editorial has been on adulticides against *Anopheles*, many of the same principles apply to mosquito control tools more generally and against a wider range of insect vectors. There is a lack of specific guidance on the evaluation of products targeting *Aedes* mosquitoes, partly due to the fact that the available methods to monitor *Aedes* populations are insufficient, and so measuring entomological impact is difficult. Products or control efforts aimed at *Aedes* are also very rarely used in isolation, necessitating the evaluation of integrated approaches, and making the link between bioassay results and predicted impact more complicated. It is a similar story for products used in larval control and for commercial products such as emanators or spatial sprays as well as newer classes under evaluation.

The pipeline for new vector control tools has never been richer, with a variety of product types and vector control strategies under evaluation for both epidemiological and entomological impact. This pipeline is an achievement to be celebrated, but all of these approaches will require testing methods to measure their efficacy and predict or directly determine entomological and epidemiological impact. The same rigorous approach can be applied to other phases of product development, for example in the screening of new AIs or in formulation development. In all these areas we recommend that the same considerations be taken in developing and validating the required standardised testing methods, including clearly defining the relevant endpoints, standardising or characterising inputs and testing parameters, and being clear on how to analyse, interpret and report data in order to use the results to make robust, evidence-based decisions.

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Reviewing the WHO Tube Bioassay Methodology: Accurate Method Reporting and Numbers of Mosquitoes Are Key to Producing Robust Results

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Simple Summary: The "WHO susceptibility bioassay" is a method from the World Health Organization used to monitor the resistance to insecticides in mosquito populations. This method was first developed in the 1960s and has undergone multiple changes since then. While these changes may appear minor, the numerous iterations of the test procedures leave some parameters open to interpretation, and changes to methodology may affect results. To address this, we reviewed the published test procedures for this method and the published literature which cited this method to see where the method could be optimized and exactly how people were reporting their use of this method. This revealed that the method is not being carried out consistently, and that the most up to date iterations of the test procedures are not always referenced. To address this, recommendations on the referencing and reporting of this method were developed. Alongside this literature review, we detail experimental work that explored whether altering parameters with room for interpretation in the test procedures could impact bioassay results. From the results, suggestions have been made to tighten certain parameters to avoid inaccurate measures of insecticide resistance. Closer adherence to the method and tightened parameters should lead to the generation of more robust data from the bioassay.

Abstract: Accurately monitoring insecticide resistance in target mosquito populations is important for combating malaria and other vector-borne diseases, and robust methods are key. The "WHO susceptibility bioassay" has been available from the World Health Organization for 60+ years: mosquitoes of known physiological status are exposed to a discriminating concentration of insecticide. Several changes to the test procedures have been made historically, which may seem minor but could impact bioassay results. The published test procedures and literature for this method were reviewed for methodological details. Areas where there was room for interpretation in the test procedures or where the test procedures were not being followed were assessed experimentally for their impact on bioassay results: covering or uncovering of the tube end during exposure; the number of mosquitoes per test unit; and mosquito age. Many publications do not cite the most recent test procedures; methodological details are reported which contradict the test procedures referenced, or methodological details are not fully reported. As a result, the precise methodology is unclear. Experimental testing showed that using fewer than the recommended 15–30 mosquitoes per test unit significantly reduced mortality, covering the exposure tube had no significant effect, and using mosquitoes older than 2–5 days old increased mortality, particularly in the resistant strain. Recommendations are made for improved reporting of experimental parameters

Keywords: insecticide resistance; resistance monitoring; method validation; WHO tube

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1. Introduction

Test procedures are published by the World Health Organization (WHO) on the use of the WHO insecticide susceptibility bioassay (or WHO tube bioassay) to monitor the resistance in adult mosquitoes to a range of insecticides commonly used for mosquito control [1]. Resistance monitoring using this approach relies on the collection of wild female adult mosquitoes or wild larvae, which are then reared to adulthood in a test facility. They are then exposed to a discriminating concentration (DC) of insecticide on a treated filter paper so that their knockdown and mortality can be scored.

The WHO tube bioassay is a simple direct response-to-exposure test. The test kit was developed in 1958 to test for the emergence of resistance to organochlorine and organophosphate insecticides following widespread resistance to organochlorine insecticides [2]. It was designed to expose a defined number of adult mosquitoes of known ages and physiological statuses to an insecticide impregnated on a filter paper for a standard exposure time (1 h).

While the WHO test procedures provide parameters for some key environmental conditions which should be kept constant while carrying out the bioassay, during insecticide resistance monitoring there are multiple potential sources of (non-resistance-associated) variability, which can influence the result of the bioassay. During a recently concluded formal WHO multicenter study to establish species-specific discriminating concentrations and procedures for new and existing insecticides, WHO tube and WHO bottle assays (an adaptation of the Centre for Disease Control (CDC) bottle bioassay developed to align end points with those of the WHO tube test) were used to generate concentration response data in multiple testing centers for a range of insecticides and multiple *Anopheles* and *Aedes* species, so that DCs could be established and validated. Within this extensive dataset, a substantial degree of variation was seen both within and between centers performing replicate assays using the same standardized methodologies [3].

One of the sources of variability in data generated when using the WHO tube bioassay methodology is the mosquitoes being tested. When using the method in the field to screen for resistance in the target population of an intervention, wild-caught adult mosquitoes should be used so that any differences in susceptibility may more closely reflect the changes in intrinsic resistance level seen for a particular intervention, and the sampled populations will be representative samples of the wild vector population in terms of age distribution and genetic variability. However, the age distribution, blood feeding status, nutritional status, and gravidity will vary between samples and potentially reduce the comparability of the results between tests and between sites. This differs from the mosquito populations that DCs are established on. Moreover, while operators using this methodology should ensure that they follow the guidance for the selection of mosquitoes for testing, it is still possible to accidentally include mosquitoes that fall outside these parameters (e.g., older than 2–5 days, males, partially blood-fed) when testing with wild-caught mosquitoes. Instead of wild-caught mosquitoes, F_1 progeny of wild-caught mosquitoes can be used; although this requires facilities to rear and test the mosquitoes, there is greater control over the rearing conditions than for wild-caught mosquitoes.

Larval rearing conditions in laboratories have been shown to have an impact on bioassay results. Overcrowding or poor diet reduce insecticide tolerance by reducing size and fitness, for example [4,5]. Any impact on longevity because of larval rearing conditions could impact the outcome on mosquito survival. Poor mosquito survival could lead to high control mortality and more discarded tests (control mortality > 20%); this will affect the feasibility of testing. Larval rearing may also be more important for insecticides such as chlorfenapyr, where the effect of the compound is impacted by metabolism, though the correlation between longevity and size is not always positive [6]. In contrast, when the WHO tube bioassay is employed for research, a well-characterized (and ideally susceptible, so that resistance levels do not have to be maintained) laboratory strain can be used alongside the mosquito strain of interest. The benefit of this is that the researcher knows the rearing conditions of their laboratory strain and the resistance status and background

of the strain, while this is not the case for field-caught mosquitoes, this well-characterized susceptible reference strain can be tested alongside as a comparator.

The effect of time-of-day of testing on bioassay results is not well-explored in the literature, but *Anopheles* typically bite at night when they may be more metabolically active, and so susceptibility testing may yield differential results if conducted during the day than during the night. Most testing is carried out during the day, so this is unlikely to be a significant source of variability of the data currently being generated using the WHO tube bioassay method. It is good practice, however, to conduct resistance-monitoring assays at the same time of day each time and report the testing time alongside the data to aid interpretation, as it has been shown that time of day can impact metabolic detoxification and insecticide resistance in *Anopheles gambiae* [7]. Chlorfenapyr (while not validated for use in the WHO tube bioassay) is also strongly affected by a temperature of <25 °C [3].

Mosquito age has been shown to affect insecticide resistance, with mosquitoes older than 10 days post-emergence showing increased susceptibility to insecticides [8]. As well as age of mosquito, the nutritional status of adults can also affect the response to insecticide exposure. Machani et al. showed that the ingestion of a blood meal increased insecticide susceptibility [9]. Further to this, it has also been shown that lowering the temperature during insecticide susceptibility testing below the recommended 27 ± 2 °C can strongly affect insecticide tolerance [10]. As part of a study conducted in a Ugandan field insectary, which lacked environmental controls but where temperature and humidity were monitored, a strong and highly statistically significant decline in *A. gambiae* mortality was detected as humidity increased [11]. In light of this, it is important to be as consistent as possible when performing susceptibility bioassays and, where it is not possible to control the conditions fully, at least to understand the effect external factors can have on the outputs from this testing and report the environmental conditions alongside the data so that the results can be interpreted accordingly.

The WHO test procedures for monitoring Insecticide susceptibility have been reviewed and updated multiple times since their original publication in 1958, and some methodological details have changed between versions. We therefore set out to review the current literature to identify which test procedures are being referenced when using this method, what data and methodological detail is being reported when this method is used, and where data gaps lie for this methodology. We aimed to achieve this by looking for parameters in the test procedures which leave room for interpretation and using a literature search to explore how these parameters can influence the results of the bioassay. Parameters which were not clearly defined or supported with evidence and where evidence is not already available in the literature were chosen to be explored experimentally. In doing this, we hope to suggest additional guidance on the optimum method for performing the WHO tube bioassay, as well as the key information required for the reporting of insecticide resistance data, thus producing more robust data and reporting it in a way that supports more meaningful interpretations.

2. Materials and Methods

2.1. Test Procedures Review

Thirteen WHO documents containing details outlining how to perform the WHO tube bioassay or the rationale behind the bioassay parameters, both published test procedures and meeting reports were reviewed to extract specific methodological details outlined in the test procedures. For each of these documents, the specifics and justifications for the methodological details outlined below were noted for each document and then compared.

- Mosquito age
- Number of mosquitoes per test unit
- Number of mosquitoes required per treatment tested
- Tube remaining still during exposure or being agitated
- Vertical or horizontal orientation of the test unit
- Exposure time

- Insecticide concentrations
- Carrier oil
- Details on insecticide-treated paper use, preparation (if not purchased from the WHO site in Malaysia), and storage
- Insecticide class specific recommendations for carrying out the bioassay
- Any recommendations for behavioral assessment
- Species specific recommendations for carrying out the bioassay
- Interpretation of results
- Details on inclusion of positive and/or negative controls
- Use of synergists
- Criteria for scoring knockdown/mortality

2.2. Literature Review

In January 2021, a literature search was performed on PubMed and BioMed Central databases using the search terms "mosquito", "WHO", and "tube" on both PubMed and BioMed Central. Results were sorted by relevance; 49 results were returned in PubMed, and 1483 results (of which a sample of the first 740 (~50%) were screened as a representative sample) were returned in BioMed Central using these search terms. Of the publications selected for inclusion in this way, 35 came from PubMed and 57 from BioMed Central. Duplicates were excluded, and further publications were excluded to allow for the comparison of methodology and mortality data between publications, including:

- Incorrect referencing of WHO tube bioassay test procedures (e.g., an academic publication or other WHO documentation which did not provide a fully outlined protocol for the WHO tube bioassay)
- Number of mosquitoes used for bioassay was not reported—the power of the study cannot be determined, so the statistical significance of the data is unknown and therefore not comparable to other studies
- Mosquito age was not reported—the data could be generated on mosquitoes outside the recommended testing age range and so the study is not necessarily comparable to other studies.
- Non-standard insecticide was used in the exposure tube for example use of technical grade chlorfenapyr, which had no DC established in the test procedures (however, a tentative DC was outlined in the 2016 test procedures [1]); use of a formulated IRS product; or a version of the bioassay adapted to use an LLIN.

A second search was performed using "mosquito", "WHO", "susceptibility", and "bioassay" to account for alternative ways of referring to the bioassay, but no additional publications were identified. This left 61 publications to be included in the analysis, as detailed in Figure 1.

The same information was then extracted from the identified publications as was extracted from the test procedures as well as:

- Insecticide class-specific recommendations for carrying out the bioassay.
- Species-specific recommendations for carrying out the bioassay.
- Interpretations of results.
- If field mosquitoes were used, whether a susceptible reference strain was tested alongside.
- Sample size.
- Any additional methodological details.

Details of all identified publications are given in Supplementary File S1.

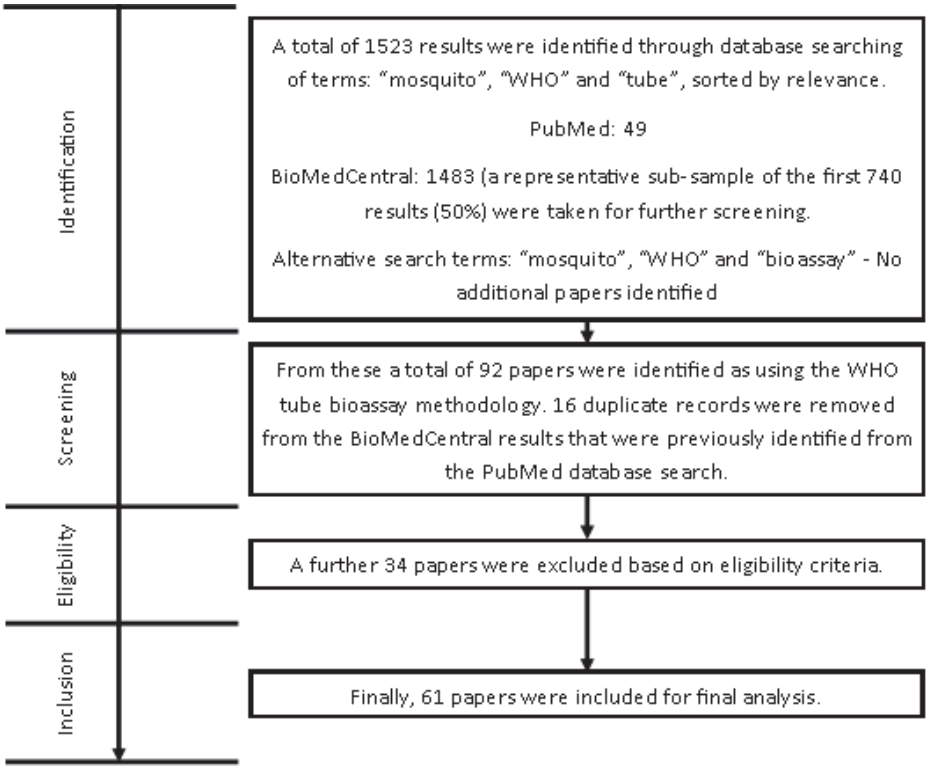

Figure 1. Flow diagram describing methodology of the literature review.

2.3. Experimental Investigation of Parameters

2.3.1. Mosquito Rearing

Mosquito colonies were maintained as described by Williams et al., in the LITE facility at the Liverpool School of Tropical Medicine (LSTM) [12]. Insectary conditions were maintained at 26 ± 2 °C and 70 ± 10% relative humidity (RH), with a L12:D12 h light: dark cycle and a 1 h dawn and dusk. Larvae were reared in purified water and fed ground TetraMin® tropical flakes (Tetra U.S., Blacksburg, VA, USA), adults were provided continuous access to a 10% sucrose solution, and adult females were given access to blood using a Hemotek membrane feeding system (Hemotek Ltd., Blackburn, UK). Two well-characterized laboratory strains of mosquito were used as representative populations, one susceptible and one resistant to commonly used insecticides. *A. gambiae* Kisumu is a reference insecticide-susceptible strain originally from Kisumu, Kenya, reared at LSTM since 1975, and *A. gambiae* Tiassalé 13 is a resistant *Anopheles* strain which was colonized from Tiassalé, Côte d'Ivoire and has been reared at LSTM since 2013. Kisumu has no selection procedure and so is susceptible, whereas Tiassalé 13 is selected with a 1 h exposure to 0.05% deltamethrin and shows high resistance to pyrethroids, which is mediated by both target sites 1014F *kdr* and *ace-1*, and metabolic resistance, which is mediated by several cytochrome P450s.

2.3.2. WHO Tube Bioassay Testing

A WHO holding-tube and its exposure tube pair are referred to as a 'test unit'. Test units using mosquitoes from the same cohort are technical replicates of each other. Test units using mosquitoes from different cohorts are referred to as biological replicates. Three

biological replicates, each made up of 2 negative control test units and 12 insecticide test units, were carried out for each experiment. There were 2 test units per treatment within a biological replicate, which were technical replicates of each other. The WHO tube bioassay 4 was used with some adaptation to allow investigation of 2 individual parameters:

- The number of mosquitoes per test unit—this was chosen as ~10% of publications identified in the literature review reported using less than the recommended range of mosquitoes as outlined in the test procedures, and a further ~13% did not report this information.
- Covered or uncovered exposure tube—this was chosen as it is mentioned only in the test procedures from 2016.
- Mosquito age—this was chosen as ~44% of publications identified used mosquitoes of the incorrect age for the test procedures they referenced.

Other factors, such as orientation of the test unit, sample size required per treatment, and sample size required per control, were either already clearly defined in the current test procedures or supported by previously published literature.

Grade 1 Whatman filter papers of size 15 × 12 cm were coated with 0.043% permethrin dissolved in silicone oil for Kisumu testing (an LC_{50} determined from previous work in the department by WHO tube bioassay for the Kisumu strain) and 0.75% (WHO recommended discriminating concentration for *Anopheles* [1]) permethrin for Tiassalé 13 testing. The 0.043% papers were made in the LITE laboratories, whereas the 0.75% papers were purchased from WHO Malaysia (Universiti Sains Malaysia, Penang, Malaysia). Permethrin was chosen as it is a heavily used insecticide for profiling, with a well understood mode of action and established resistance mechanisms in the Tiassalé 13 strain.

To investigate the effect of the number of mosquitoes per test unit and compare the results from covered and uncovered tubes in parallel, the experimental layout for a single replicate is outlined in Table 1. Additional technical replicates of the tubes containing fewer mosquitoes were conducted to ensure equivalent numbers of mosquitoes were screened per treatment and to nullify the potential bias of a smaller sample size influencing the mortality estimate.

Table 1. Experimental outline of a single biological replicate to investigate the effect of covered vs. uncovered exposure tubes and number of mosquitoes per test unit. The test concentration was 0.043% for *Anopheles gambiae* (Kisumu, susceptible) testing and 0.75% for *An. gambiae* (Tiassalé 13, resistant) testing. Mosquito age was 2–5 days for both strains and all treatments.

Test Unit	Treatment	Number per Test Unit	Covered/Uncovered
Negative Control 1	Silicone oil only	25	Uncovered
Negative Control 2	Silicone oil only	25	Uncovered
Uncovered 1	Permethrin	25	Uncovered
Uncovered 2	Permethrin	25	Uncovered
Covered 1	Permethrin	25	Covered
Covered 2	Permethrin	25	Covered
30 per test unit 1	Permethrin	30	Uncovered
30 per test unit 2	Permethrin	30	Uncovered
20 per test unit 1	Permethrin	20	Uncovered
20 per test unit 2	Permethrin	20	Uncovered
15 per test unit 1	Permethrin	15	Uncovered
15 per test unit 2	Permethrin	15	Uncovered
10 per test unit 1	Permethrin	10	Uncovered
10 per test unit 2	Permethrin	10	Uncovered

Mosquitoes were exposed in test units for 1 h at 26 ± 2 °C and 70 ± 10% RH and then transferred back to holding-tubes post-exposure and held in the same conditions for 24 h at which point their mortality was scored. Data from three biological replicates, each prepared independently, were used to generate the data.

To investigate the effect of mosquito age, a second experiment was completed using a single cohort of each strain, from which a subsample of 150 mosquitoes was taken at the WHO recommended testing age [3] (2–5 days), 2 days after testing age (overlapping with the recommended age range, 4–7 days), and 4 days after testing age (outside the recommended age range, 6–9 days). Mosquitoes were tested with two negative control test units and four insecticide test units. The test concentrations were 0.043% for Kisumu testing and 0.75% for Tiassalé 13 testing, as in the previous experiment. This experiment was repeated three times, each with independently reared cohorts of mosquitoes.

2.3.3. Data Analysis

Mortality was calculated as the total number of individuals knocked down or dead in a test unit as a percentage of the total number of individuals in the test unit at the end of the 24 h scoring period. If the mortality in the negative control test unit was <20% but ≥5%, then the observed mortality in each treatment test unit was corrected using Abbott's formula [13]. Where the control mortality was ≥20%, the results were discarded and the test replicate repeated.

Both datasets (age and number of mosquitoes per tube) were screened using a binomial generalized linear model (GLM), a binomial generalized linear mixed model (GLMM) with a random effect for biological replicates to account for any inter-assay variation, a binomial GLMM with a random effect for biological replicates, and a nested random effect for technical replicate to account for the intra-assay variation using the glmmTMB package in R [14]. For each analysis, the variable was treated as a factor, with 5 days used as a reference for age and 25 mosquitoes used as a reference for the number of mosquitoes in the tube. The negative controls were excluded from all analyses, as we were only testing for the influence of these factors on the deviation from the reference. A likelihood ratio test (LRT) was conducted to identify the best-fitting GLMM.

3. Results

3.1. Comparison of Test Procedures

Although the current WHO test procedures recommend a single DC assay to detect resistance [3], the WHO tube bioassay method initially recommended a concentration response experiment [15]. Field-caught blood-fed females were used, with 15–25 mosquitoes per test unit using a series of four concentrations, which should lie on a range giving 0–100% mortality with four replicates per concentration for a total of 200 mosquitoes per test concentration. If a population of mosquitoes was highly resistant, the exposure time was increased by 1 h until significant mortality was seen. This method continued to be recommended until 1970, when the method changed from recommending four concentrations to only two concentrations, with the lowest concentration to be tested first with a range of exposure times [16]. At a WHO meeting in 1976, this was changed again to a single concentration known as the discriminating concentration [17]. These updates were made in order to simplify the bioassay to fewer test concentrations for the growing list of insecticides which required resistance monitoring.

The test kit itself was initially eight exposure tubes marked with a red dot, two control tubes marked with a green dot, and ten holding-tubes also marked with a green dot (see Figure 2). Moreover, it was specified that the impregnated papers could initially be reused up to 20 times, and the test kit had to be oriented with the mesh screen facing up during exposure.

Several meetings were held between the years 1958 and 1992 to discuss the changes to the methodology to address increased resistance to insecticides and to add new insecticide classes (See Table 2 for a list of meeting reports) [2,15–20]. The test procedures were then updated in 1998 following a multicenter study which recommended DCs for five pyrethroid insecticides [21]. This update also included some methodological changes. Single discriminating concentrations were provided for both the newly added pyrethroid insecticides and the organochlorines, organophosphates, and carbamates. Minor adjustments were made

to the test kit itself to reduce it from a 20-test unit kits to a 12-test unit kit consisting of five exposure tubes marked with red dot, two control tubes marked with a green dot, and five holding-tubes also marked with a green dot. Testing a minimum of 100 mosquitoes (4–5 replicates of 20–25 mosquitoes) per concentration was recommended. Mosquitoes for testing were now required to be 1–3-day-old non-blood-fed females. These mosquitoes were either F_1 progeny from larval collections or field-caught mosquitoes. The temperature range of 25 ± 2 °C and 70–80% RH was specified. Insecticide-treated papers were only able to be used 5 times, as opposed to the previously recommended 20. The vertical orientation of the test tubes during performance of the bioassay was further justified in these test procedures, as horizontal positioning avoids the knockdown and recovery of mosquitoes, since knocked down mosquitoes would lie on treated paper instead of the untreated mesh-end of the test unit and so still be exposed to the insecticide. This would increase the exposure of the mosquito, and the exposure route may not be through the tarsi of the mosquito [21].

The WHO test procedures were then updated again in the 2006 "Guidelines for testing mosquito adulticides for residual indoor spraying and treatment of mosquito nets". Little changed between the 1998 version of the test procedures and this version; the recommended humidity changed from 70–80% RH to 80 ± 10%, and 2–5 day old mosquitoes were specified instead of the previous 1–3 day old [22]. Then, in 2013, the "Test procedures for insecticide resistance monitoring in malaria vector mosquitoes" was published. Minor adjustments were made to the test kit itself; the new 12-test-unit kit consisted of four exposure tubes marked with a red dot, two control tubes marked with a yellow dot, and six holding-tubes also marked with a green dot. At least 120–150 active 3–5-day old female mosquitoes were recommended to be exposed in batches of 20–25, ideally with at least 100 per insecticide and 50 as controls [23].

The most recent update to the test procedures came in 2016 [25]. These procedures aimed to provide a stronger focus on producing operationally meaningful data and so introduced resistance intensity (RI) assay testing (using 5× and 10× the pyrethroid DC) and pyrethroid-PBO synergist bioassays as additional testing alongside the standard WHO insecticide susceptibility bioassay. Again, slight changes were made to the WHO tube bioassay protocol. The temperature and humidity changed to 27 ± 2 °C and 75 ± 10% RH, and it was recommended that the test units be "placed in an area of reduced lighting or covered with cardboard discs". This was supposed to reduce the light intensity and discourage mosquitoes resting on the mesh. There was also an additional piece of WHO documentation in the 2016 "Monitoring and managing insecticide resistance in *Aedes* mosquito populations Interim guidance for entomologists", which was published as part of the response to the Zika epidemic. However, there were no methodological differences in performance of the bioassay from the previously published 2016 procedures [25]. The same methods are thus recommended for *Aedes* spp. as for *Anopheles* species.

Table 2. Summary of the review of historic versions of the World Health Organization (WHO) tube bioassay guidelines. * Initial baseline dose response for population generated with range of concentrations. Subsequent testing uses four concentrations along this range and two controls. Testing is performed in duplicate. ** Mortality in a negative control over 20% is unsatisfactory; testing should be repeated. A section to help with interpreting dose response curves is also introduced. *** Initial baseline preliminary test performed with full range, then concentrations selected for baseline assessment. Subsequent routine checks used a concentration which is double the concentration that has consistently given complete kill in successive tests. Exposure time can be increased for exceptionally insensitive populations. (2, 4 or 8 h) **** Only concentrations provided. Lowest concentration tested first with range of exposure times (30 min, 1, 2 and 4 h). ***** Introduction of a discriminating dose. ****** Piperonyl butoxide (PBO) synergism bioassay method included. Table is reproduced in Supplementary File S1.

Title and Year	Mosquitoes Per Tube	Mosquito Age	Mosquito Physiological Status	Temperature	Humidity	Orientation	Insecticides	Number per Control	Number per Treatment	Lighting	Paper Usage
7th Report of the Expert Committee on insecticides (1957)	No methodological details contained within this report. Refers to the methods of "Busvine and Nash" and "Fay et al." as possible test methods to be used for detecting and measuring resistance. No references given for these papers.										
5th Report of the Expert Committee on insecticides (1958) *	20–25	Not specified	Recently fed. Or mix of unfed and fed	Does not exceed 30 °C	Not Specified	Vertical	DDT and Dieldrin	Not specified	200	Moderate diffuse illumination.	Up to 20 times and up to weeks after opening of package Store in cool place do not refrigerate.
10th Report of the Expert Committee on insecticides (1960) **	15–25	Not specified	Recently fed. Or mix of unfed and fed	Does not exceed 30 °C	Not Specified	Vertical	DDT and Dieldrin	Not specified	200	Moderate diffuse illumination.	Up to 20 times and up to weeks after opening of package Store in cool place do not refrigerate. Expiry on package presupposes that the packages are kept sealed.
13th Report of the Expert Committee on insecticides (1963) ***	at least 15	Not specified	Recently fed. Or a mix of unfed and fed	Does not exceed 30 °C	Not Specified	Vertical	DDT, dieldrin, malathion, fenthion	Not specified	200	Moderate diffuse illumination.	Up to 20 times and up to weeks after opening of package. Store in cool place do not refrigerate. Expiry on package presupposes that the packages are kept sealed.
17th Report of the Expert Committee on insecticides (1970) ****	15–25	Not specified	Recently fed. Or mix of unfed and fed	Does not exceed 30 °C	Not Specified	Vertical	DDT, dieldrin, malathion, fenthion, OMS-33	Not specified	200	Holding tubes should be kept post exposure in a secluded shaded place.	Up to 20 times and up to weeks after opening of package. Store in cool place do not refrigerate. Expiry on package presupposes that the packages are kept sealed.

Table 2. *Cont.*

Title and Year	Mosquitoes Per Tube	Mosquito Age	Mosquito Physiological Status	Temperature	Humidity	Orientation	Insecticides	Number per Control	Number per Treatment	Lighting	Paper Usage
22nd Report of the Expert Committee on insecticides (1976) *****	15–25	Not specified	Recently fed. Or mix of unfed and fed	Does not exceed 30 °C	Not Specified	Vertical	DDT, dieldrin, malathion, fenitrothion, fenthion, propoxur	Not specified	200	Holding tubes should be kept post exposure in a secluded shaded place.	Up to 20 times and up to weeks after opening of package Store in a cool place do not refrigerate. Expiry on package presupposes that the packages are kept sealed.
5th Report of the WHO Expert Committee on Vector Biology and Control: Resistance of vectors of disease to pesticides (1980)	Not specified	Not specified	Not specified	~25 °C	Not Specified	Vertical	DDT, dieldrin, malathion, fenitrothion, chiorphoxim, permethrin, decamethrin, propoxur	Not specified	Not specified	Not specified.	Not specified.
10th Report of the WHO Expert Committee on Vector Biology and Control: Resistance of vectors of disease to pesticides (1986)	Not specified	Not specified	Not specified	Not specified	Not Specified	Vertical	chiorphoxim, DDT, deltamethrin, dieldrin, fenitrothion, malathion, permethrin, propoxur	Not specified	Not specified	Not specified.	Can be refrigerated so long as boxes are fully sealed.
15th Report of the WHO Expert Committee on Vector Biology and Control: Vector Resistance to Pesticides (1992)	Not specified	Not specified	Not specified	Not specified	Not Specified	Vertical	DOT, dieldrin, fenitrothion, fenthion, malathion, propoxur, lambda-cyhalothrin, permethrin, deltamethrin	Not specified	Not specified	Not specified	Not specified.
Report of the WHO Informal Consultation Test Procedures for insecticides Resistance Monitoring in Malaria Vectors, sio-Efficacy and Persistence of insecticides on Treated Surfaces (1998)	20–25	1–3 days	Unfed females	25 ± 2 °C	70–80%	Vertical	permethrin, deitamethrin, lambda-cyhalothrin, cyfluthrin, etofenprox, DDT, dieldrin, malathion, fenitrotion, propoxur, bendiocarb	Not Specified	min 100, 4–5 replicates of 20–25	Not specified	Up to 20 times.

Table 2. *Cont.*

Title and Year	Mosquitoes Per Tube	Mosquito Age	Mosquito Physiological Status	Temperature	Humidity	Orientation	Insecticides	Number per Control	Number per Treatment	Lighting	Paper Usage
Guidelines for Testing Mosquito Adulticides for indoor Residual Spraying and Treatment of Mosquito Nets (2006)	20–25	2–5 days	Unfed females	25 ± 2 °C	70–80%	Vertical	Not Specified	Not Specified	min 100, 4–5 replicates of 20–26	Not specified	Not more than 5 times.
Test Procedures for insecticide resistance monitoring in malaria vector mosquitoes (2013)	20–25	3–5 days	Unfed females	25 ± 2 °C	80% ± 10%	Vertical	Dieldrin, DDT, malathion, fenitrothion, propoxur, bendiocarb, permethrin, deltamethria, lambdacy-halothrin, cyfluthrin, etofenprox	50 per control, 2 replicates of 25	120–150, 6 reps 20–25 of which 2 are negative controls	Not specified.	Not more than 5 times.
Test Procedures for insecticide resistance monitoring in malaria vector mosquitoes: Second edition (2016) ******	20–25	3–5 days	Unfed females	27 ± 2 °C	75% ± 10%	Vertical	Bendiocarb, carbosulfan, propoxur, DDT, dieldrin, fenitrothion, malathion, pirimiphos-methyl, alpha-cypermethria, cyfluthrin, deltamethrin, etofenprox, lambda-cyhalothrin, permethrin, PBO	50 per control, 2 replicates of 25	120–150, 6 reps 20–25 of which 2 are negative controls	Tubes must be of places in anarea of reduced lighting or covered with cardboard discs.	Not more than 5 times.

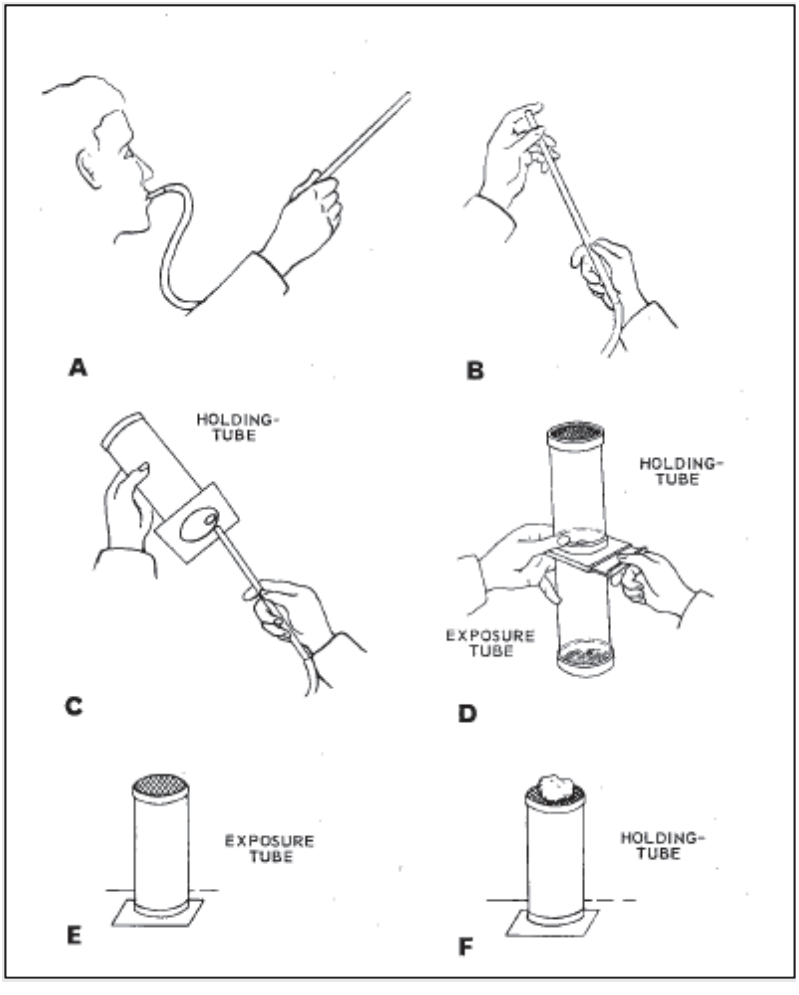

Figure 2. Original World Health Organization (WHO) tube method as outlined in the "8th Report of the Expert Committee on Insecticides" [24], reproduced with permission of Rajpal Singh Yadav, WHO. (**A**) Collect test mosquitoes using a mouth aspirator. (**B**) Mosquitoes should be collected in batches of no more than 10. (**C**) Test mosquitoes are gently transferred to the holding-tubes until they number 20–25 per tube. (**D**) The exposure tube is attached, and the slide is opened. Mosquitoes are then gently blown from the holding-tube to the exposure tube. The holding-tube is detached and set aside (**E**) The exposure tubes are left standing upright for 1 h during the exposure. (**F**) Mosquitoes are transferred back to the holding-tube by reversing the process described in C. The holding-tube is set upright, and a pad of wet cotton wool is placed on top. Tubes are held for 24 h, at which point mortality counts are made.

The criteria for scoring knockdown and mortality in this bioassay have remained unchanged. However, there is room for interpretation around what is or is not a knocked down mosquito. When testing pyrethroids with adult mosquitoes, it is common to see surviving individuals with several legs missing. These mosquitoes are still technically alive and able to fly but have clearly been impacted by the exposure. To take this into account, Hougard et al. assessed "functional mortality" alongside normal mortality scor-

ing (dead mosquitoes only). Functional mortality was defined as "including surviving mosquitoes with three legs or fewer", as it is assumed that mosquitoes with three legs or fewer would not survive in the field. From this study, considering functional mortality provided additional information as well as a better estimate of the overall killing effect of a pyrethroid insecticide [26]. However, Isaacs et al. showed that insecticide-induced leg loss had no significant effect upon either the blood-feeding or egg-laying success of exposed mosquitoes. A non-significant reduction in blood-feeding success was seen with 1-legged insecticide-exposed mosquitoes, and, while their egg laying behavior appeared to be altered, the eggs laid were fertile and hatched to larvae. We conclude that studies of pyrethroid efficacy should not discount mosquitoes that survive insecticide exposure with fewer than six legs, as they may still be capable of biting humans, reproducing, and contributing to malaria transmission [27].

3.2. Review of the Literature

Only the 1998, 2006, 2013, and 2016 test procedures were referenced in the sampled publications, with the majority referencing either the 1998 or the 2013 test procedures. However, when comparing the publication date of a journal article and the publication dates of the test procedures referenced within, over half the publications were using test procedures that were between 3 and 18 years out of date (See Figure 3).

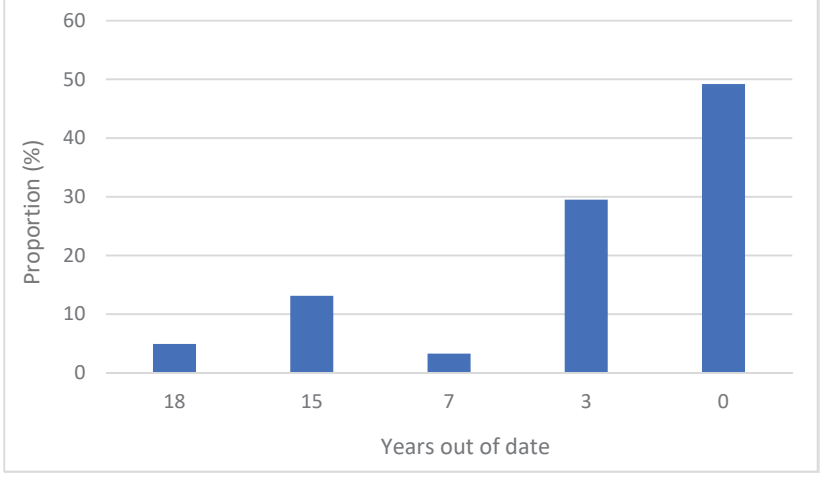

Figure 3. The number of years out of date the referenced guidelines were for a given publication in relation to the most recent guidelines available at the time of publication.

The test procedures have remained consistent since 1998 in outlining the number of mosquitoes per test unit as 20–25. When looking at the number of mosquitoes per test unit used for testing in the published literature (see Figure 4), approximately 90% were within the WHO range. Those that lay outside the range tended to use between 10 and 15 mosquitoes; these were often field studies, and so this was likely due to the limited availability of mosquitoes in the field. This was also mirrored in the number of mosquitoes used per treatment. The WHO recommend 100 per treatment, but again field studies often used less than this, which again was probably because of mosquito availability. Studies that showed numbers of mosquitoes per treatment larger than 250 were often pooled from multiple sites or multiple rounds of testing. However, 44% of the publications sampled reported "20–25" mosquitoes instead of the actual numbers used per test unit, which shows that they followed the test procedures but does not provide accurate 'n' values for a given treatment. Several papers reported using mosquitoes in the range of "15–25", "10–15", or "10" per test unit. No justification for this deviation from the WHO test procedures for this

bioassay is provided within the publication. However, it can be assumed that, due to these studies either using field-caught larvae reared to adults or F_1 larvae of field-caught adults reared to adults for their bioassay testing, they would be limited in terms of total sample size and so reduced the number per test unit to increase technical replication.

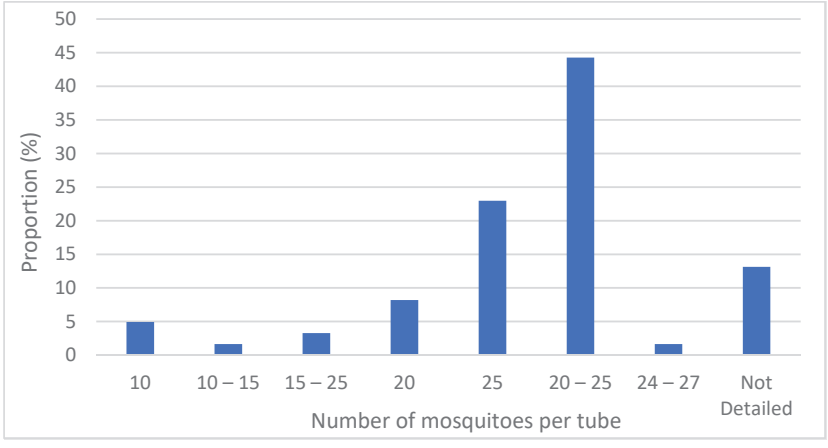

Figure 4. The number of mosquitoes used in an individual tube for publications reviewed.

Since 2013, the WHO test procedures have recommended a minimum of 50 mosquitoes to be exposed to control papers in 2 batches of 25 each alongside the 100 required per treatment. This is often not reported in the literature, with around 80% of publications not reporting this information; however, this is unsurprising, as it is a more recent addition to the WHO test procedures.

The source of the exposure papers is often not reported, with nearly 45% of publications using terminology along the lines of "papers impregnated with insecticide were used"; however, it is unclear from this whether papers were made by the researchers themselves or purchased from Malaysia. Since 1993, the WHO have provided standardized insecticide papers from their site in Malaysia, and over 40% of publications stated that their exposure papers were sourced from there. The studies which did specify the source of papers as other than from the WHO either impregnated their own exposure papers, had them made up by a partner research institute, or purchased them from a center for disease control or other public health body.

The recommended mosquito age has changed several times throughout the different iterations of the test procedures. In 1998, 1–3 days was recommended, until this was updated in 2006 to 2–5 days and again in 2013 to 3–5 days. For publications referencing the 1998 test procedures, 85% used mosquitoes older than recommended. For publications referencing the 2013 test procedures onwards, 23% used mosquitoes younger than recommended. So, 44% were using mosquitoes of the incorrect age for the test procedures they referenced (see Figure 5).

The sampled manuscripts described the results from a range of Anopheline and Culicine species, though the species were not always identified, as well as a large number of insecticides from different mode of action classes (detailed in Supplementary File S1). In instances where more than one publication tested the same combination of strain of mosquito and insecticide, we compared the data between the two publications. A total of 44% of publications used only a field strain and so data was not comparable. For the remaining publications, 38% included a susceptible *A. gambiae* (Kisumu), 13% used an unspecified laboratory strain, two publications used a susceptible *A. funestus* (FANG), and one publication used the susceptible *A. coluzzii* (N'gousso) as reference strain alongside the testing of field populations. The data for these susceptible reference strains agreed between

the publications; however, the mortality was often 100%, as the strain being tested was a susceptible laboratory strain. Three publications exposed resistant mosquito strains to discriminating concentrations to profile their resistance phenotype. Bagi et al. [28] and Williams et al. [12] both exposed Tiassalé 13 to 0.75% permethrin for 1 h; Bagi showed a 3.4% mortality 24 h post-exposure, whereas Williams et al. showed ~20% mortality when the strain was profiled in the years 2017 and 2019 [12,28]. Owusu et al. [29] also exposed Tiassalé 13 to 0.75% permethrin for 1 h and showed a mortality of 78.0%, whereas Williams et al. [12] showed approximately 5% mortality for the years 2017 and 2019.

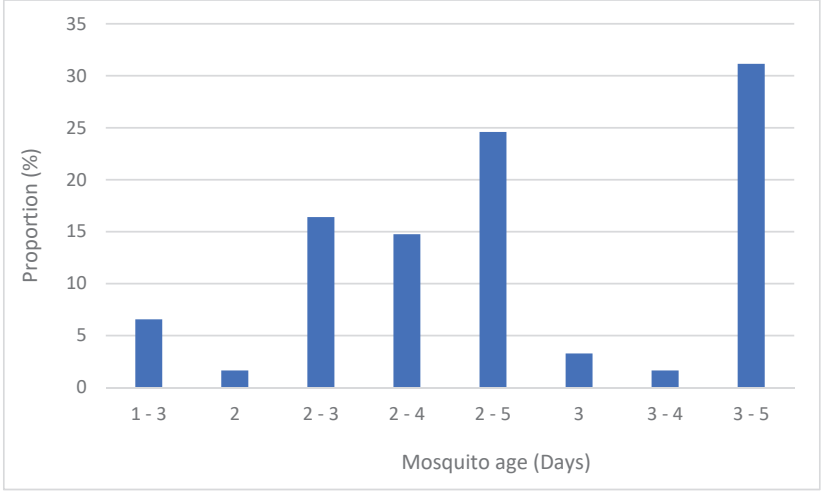

Figure 5. The age of mosquitoes tested for publications reviewed.

3.3. Experimental Investigation of Parameters

The GLMM accounting for biological effect was used to generate the effect estimate for the two variables of interest. The Kisumu strain was much more susceptible to knockdown, assessed at 60 min, when there was a reduction in the number of mosquitoes per tube with a significant reduction in knockdown for tubes with 20 (OR = 0.42, $p = 0.001$, 95% CI = 0.26–0.69), 15 (OR = 0.35, $p \leq 0.001$, 95% CI = 0.21–0.59), and 10 (OR = 0.2, $p \leq 0.001$, 95% CI = 0.11–0.36) mosquitoes. This significant reduction was still found when evaluated again at 24hrs in the tubes of 10 and 20 mosquitoes; however, this was no longer present for the tubes containing 15 mosquitoes. For the Tiassalé 13 data, the 60 min assessment also found a significant reduction in tubes containing 10 mosquitoes (OR = 0.28, $p = 0.004$, 95% CI = 0.12–0.67); however, this effect was not discernable when evaluated again at 24 h (Figure 6, Appendix A).

An additional treatment was performed with 25 mosquitoes per test unit with a cardboard disc covering the top of the tube during exposure. No significant difference was detected for this alteration in the study protocol in either the Kisumu strain (OR = 0.83, $p = 0.391$, 95% CI = 0.53–1.28) or Tiassalé 13 at 24 h (OR = 1.27, $p = 0.462$, 95% CI = 0.67–2.39).

For the Kisumu strain, the 4–7-day-old and 6–9-day-old mosquitoes showed a significant increase in mortality at 60 min knockdown. However, only the 6–9-day-old mosquitoes maintained this statistical significance when assessed at 24 h (OR = 2.46, $p \leq 0.001$, 95% CI = 1.69–3.59). The mortality assessment for the 2–5-day group's mortality increased from around 26 to 64% between assessment periods.

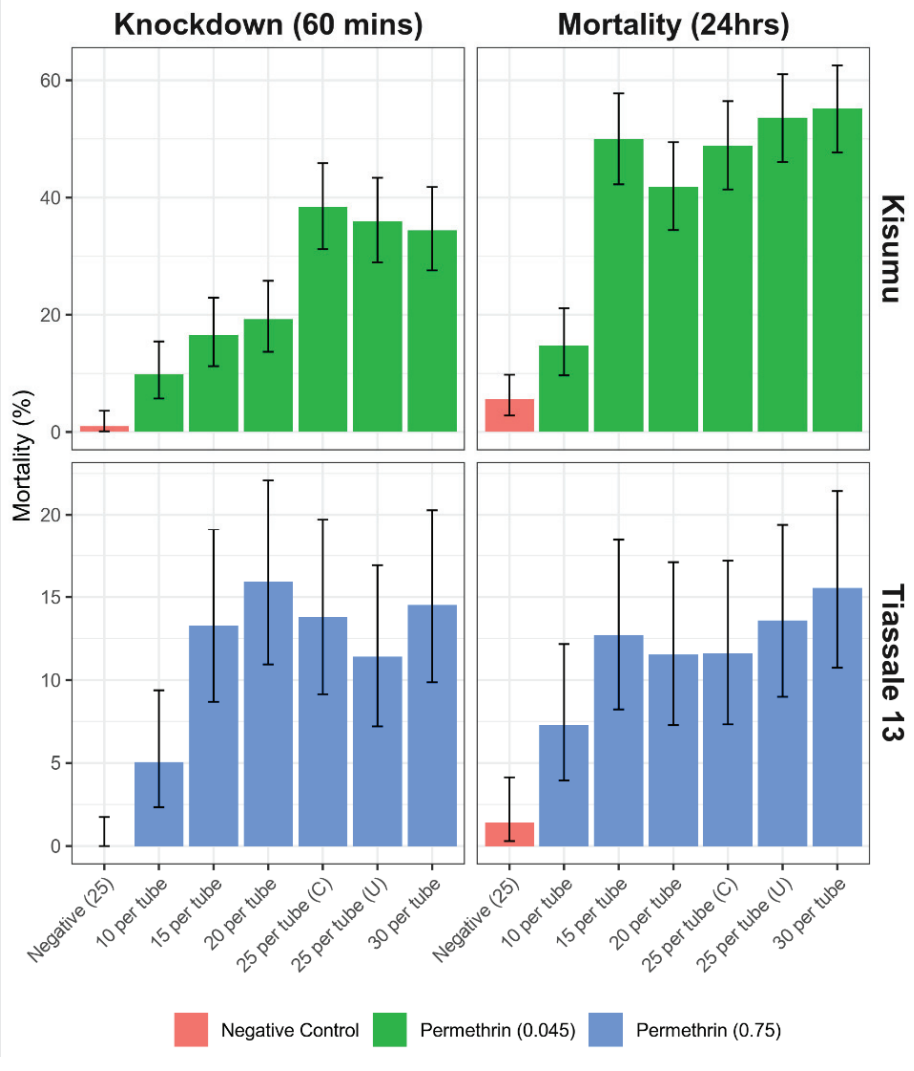

Figure 6. Bioassay data looking at the number of mosquitoes used in testing may impact the result of standardized bioassay testing for an *Anopheles gambiae* susceptible Kisumu strain and a resistant Tiassalé 13 strain. A total of 25 per tube (C) had the top of the tube covered during exposure, while 25 per tube (U) had the same conditions as the other tubes. Error bars equate to the 95% confidence intervals of the proportion.

This trend for older mosquitoes to show a greater susceptibility following exposure was also seen for the Tiassalé 13 strain (Figure 7) with the 6–9-day-old group showing an increase in mortality of ~9% points compared to the 2–5-day-old group at 60 min (OR = 2.99, $p \leq 0.001$, 95% CI = 1.64–5.46), and both the 4–7-day- and 6–9-day-old groups showing increased mortality at 24 h (Figure 7).

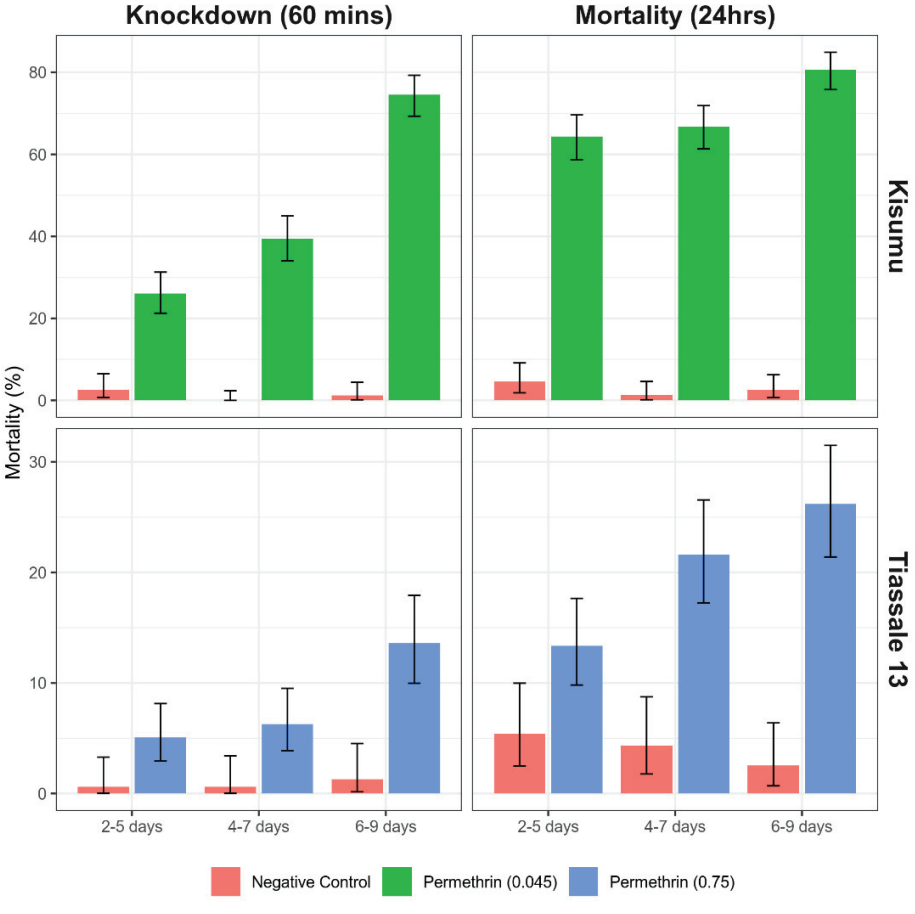

Figure 7. Bioassay data looking at how age of mosquitoes used in testing may impact the result of standardized bioassay testing for a susceptible *Anopheles gambiae* Kisumu strain and a resistant Tiassalé 13 strain. Error bars equate to the 95% confidence intervals of the proportion.

4. Discussion

It is clear from the details outlined in Table 2 that the WHO susceptibility bioassay has undergone numerous updates to its methodology since its inception. While at each stage these updates have been relatively minor, it is still possible that these could impact bioassay results, and so it is important to ensure that the most recent iteration of the test procedures is followed and referenced. However, the literature review shows that this is not always the case.

The methodological variability between the published test procedures and the way these test procedures were historically presented on the WHO website leaves the WHO tube assay for insecticide susceptibility in mosquitoes open to interpretation as to how to perform the bioassay, as well as being unclear as to what the most up to date iterations of the guidelines are. The WHO website has been updated since this review of the method began, and the relevant test procedures can now be found considerably easier (https://www.who.int/teams/global-malaria-programme/prevention/vector-control/insecticide-resistance [Accessed: 4 February 2022]). Moreover, the lack of comparable data from the published literature is due to the populations being tested being either field strains

of unknown resistance status or a susceptible laboratory strain. For the few publications where the same resistant laboratory strains were able to be compared, the mortality data was wildly different. This could be because the same strains held in different labs might in fact be vastly different from each other. This could be because of a whole host of reasons, including laboratory adaptations, contamination, selection pressure applied rearing conditions, genetic bottlenecks, and genetic drift. As a result, to optimize this bioassay, we planned to investigate the effect of mosquito age in the range of 5–10 days old, as well as the number of mosquitoes per test unit and the use of cardboard discs to cover the mesh of the exposure tube during the test, which is specified in the most recent iteration of the test procedures. These factors were chosen for investigation, as there is a lack of published literature investigating their effect on the outcome of this bioassay.

The susceptible *A. gambiae* strain Kisumu was exposed to permethrin-treated papers of a concentration expected to provide moderate mortality in an experiment to explore the effect of varying the parameters of interest when conducting the WHO tube assay. Based on three replicate tests, there was no evidence that covering the top of the exposure tubes with a cardboard disc during the exposure period had any impact on either 1 h knockdown or 24 h mortality. The rationale for the covering of the exposure tubes using in the test procedures is that it will prevent light entering through the mesh and so should discourage mosquitoes from resting on the upper mesh of the test units during exposure, which reduces their contact with the insecticide. It was not possible to assess if there was a reduction in resting on the mesh, as it was not possible to observe mosquito behavior during the exposure period, as the exposure chamber was covered by the insecticide-treated filter paper and the cardboard disc. However, due to the lack of significant difference in mortality seen in this study, we would suggest that this step appears to be unnecessary. So long as all test units are treated the same in terms of lighting, mosquitoes resting on the mesh should be consistent between test units and therefore there should have no impact on the final mortality scoring.

When varying the number of mosquitoes per test unit, mortality in this same experimental set up was unaffected by mosquito numbers between 15 and 30 mosquitoes per test unit. However, when only 10 mosquitoes were added per test unit, the 24 h mortality was significantly lower (7% compared to 50%). The same trend is not seen in the proportion of mosquitoes knocked down immediately post exposure, with knockdown being reduced in treatments with 15 and 20 mosquitoes per test unit compared to covered and uncovered treatments containing 25 or 30 mosquitoes. Knockdown thus appears to be positively correlated with the number of mosquitoes per test unit in this laboratory strain. This implies that mosquitoes are being differentially exposed during the bioassay, depending on the number of individuals within a single test unit. It is possible that, when using 10 mosquitoes in a test unit, there is enough space for all or most of the mosquitoes to rest on the door at the base or at the mesh at the top of the test unit and therefore avoid contact with the insecticide-treated paper. With more mosquitoes, there is more opportunity for this free flight to disturb resting mosquitoes within the bioassay and cause them to fly and resettle in a different part of the exposure chamber. This could then force the mosquitoes which were previously resting on a non-insecticide-treated surface to encounter the insecticide-treated filter paper instead and become intoxicated with the insecticide. The more mosquitoes in a test unit, the more likely this disturbance is to occur and, in turn, the more likely a mosquito is to become intoxicated with insecticide through more frequent contacts. We recommend that at least 15 mosquitoes are included per test unit when conducting WHO tube assays, and that, where knockdown is the entomological endpoint of interest, the number of mosquitoes per test unit is held constant between replicates and treatments. As well as this, due to the general increase in mortality seen with the addition of more mosquitoes, we would not recommend exceeding 30 mosquitoes per test unit. It is also worth noting that there was a substantial divergence in the mortality estimate for biological replicate test compared to the other three replicates. Despite the quality control measures in place, this may be due to some difference in the cohort of mosquitoes being used. It does

highlight the variability that can be introduced into the bioassay data by minor changes in parameters, even in highly controlled conditions. Such a divergent bioassay result could easily go unnoticed in the absence of technical and biological replicates.

Since this data was generated from three biological replicates with the same number of technical replicates per treatment, the sample size for each treatment differs, with three times as many mosquitoes tested in the 30-mosquitoes-per-test-unit treatment as in the treatment with 10 mosquitoes. To account for this difference, an additional biological replicate was carried out to equalize the sample size for each treatment to ~180. With this additional replication, the trends seen did not change. The only significant change was that the 24 h mortality for the 10-per-test-unit treatment increased from 7 to 15%. The variability of the results within each treatment was either unaffected or reduced with increased replication, showing the value of maximizing both test unit replicates and mosquito 'n' values when generating data using the WHO tube assay. For the Tiassalé 13 strain in the 15-per-test-unit treatment, the variability was significantly higher (Figure 7); however, this is due to the large intraspecific variation between the technical replicates in one of the biological replicates. It is possible that using more test units with fewer mosquitoes could result in a lower variability than fewer test units with larger numbers, and so, in settings where mosquitoes are less available, it may be beneficial to divide the cohort up into multiple smaller batches with more test units. Where possible, we would recommend increased replication with different cohorts of the same mosquito population to increase the sample size to at least the WHO recommended 100 mosquitoes per insecticide treatment and 50 mosquitoes per control. We also would not recommend using any more than 30 mosquitoes per test unit, as there is no data available, that we are aware of, to support it, and we can see no logistical reason for using more than this number per test unit.

Repeating the experiment with the insecticide-resistant Tiassalé 13 exposed to permethrin-treated DC papers with results based on three replicates showed similar trends to those using Kisumu. Covering the exposure tube during the period of exposure again had no significant effect on knockdown or mortality relative to the uncovered test units. Mosquito numbers between 15 and 30 mosquitoes per test unit did not affect knockdown or mortality, but again the treatment with only 10 mosquitoes per test unit resulted in lower mortality. The effect size of the number of mosquitoes per test unit seemed to be smaller than in the susceptible Kisumu strain, and a larger sample size was needed to be able to detect a difference. Further replicates of the experiment to ensure equal '*n*' values for the number of mosquitoes tested per treatment did not affect the trends of results; the variability was reduced, though not to the same extent as it was in the Kisumu experiment. This replicated result, even with a DC assay with insecticide-resistant mosquitoes, which are more field relevant than an old laboratory colony, supports the recommendation to use a minimum of 15 mosquitoes per test unit but that covering the exposure tube does not have an effect.

When investigating the effect of mosquito age at the time of testing, we found that mosquitoes both 2 and 4 days older than the recommended testing age (2–5 days) show an increased susceptibility to permethrin. This increased susceptibility is seen at 6–9 days old for Kisumu and 4–7 days old for Tiassalé 13. This difference could be due to the increased fitness cost caused by resistance mechanisms in the Tiassalé 13 strain compared with the susceptible Kisumu strain. This supports previous findings that mosquitoes aged 10 days and above show an increased susceptibility to insecticides [8,9], but there has previously been little data on mosquitoes aged between 5 and 10 days post-eclosion. While investigations of the effect of insecticides on malaria transmission focus on older female *Anopheles* which are the vectors of malaria, it is useful for other testing purposes to know across what age range mosquitoes can be used for testing and still produce the same result. When monitoring a population for the emergence of resistance, it is important that variables including ag, and mosquito density, as discussed above, are held constant to allow robust comparisons between test replicates and to allow true changes in test results over time to be identified. The data from this study suggest that, when performing a WHO tube assay, the recommended testing age of 2–5 days should be adhered to.

Intertest variability (between biological replicates) was generally larger than or similar to intra-test variation (between test units within a biological replicate) for both strains. It is possible that using more test units with fewer mosquitoes could result in a lower variability than fewer test units with larger numbers, and so, in settings where mosquitoes are less available, it may be beneficial to divide the cohort up into multiple smaller batches with more test units, within the limits of 15–30 mosquitoes discussed above.

5. Conclusions

While this study uses only one insecticide and one mosquito species, the insecticide chosen is a heavily used insecticide for profiling with a well-understood mechanism of action and effect on mosquito populations. The strains tested are also well-established and well-characterized laboratory strains, one of which is wholly susceptible and another of which is highly resistant. With variable results seen for this combination of insecticide and mosquito, it is possible that even more variable results would be seen with a more moderately resistant strain and with novel or less well-understood mode of action with changes to the investigated parameters.

As a result, we make the following recommendations for this bioassay method:

1. Better reporting of the conditions that a bioassay is carried out under, including information on:
    a. Holding/exposure temperature
    b. Holding/exposure humidity
    c. Source of insecticide-treated papers
    d. Expiry date or batch number
    e. Reporting of negative control data
    f. Reporting of total N per treatment
    g. Reporting of number of mosquitoes per test unit.
2. All bioassay testing should be carried out with WHO tubes positioned vertically, as stated in the test procedures, to avoid increased contact with the insecticide-treated surface from knocked down mosquitoes in a horizontally oriented test unit.
3. A minimum of 15 and a maximum of 30 mosquitoes should be tested per test unit.
4. Use of a characterized reference strain alongside bioassay testing of field strains is recommended where possible.
5. Cardboard discs to cover exposure tubes do not appear to be required, and this step could be removed from the test procedures. However, for consistency of results and methodological practice, it would be good to continue this until more comprehensive data is generated on the effect of light intensity on bioassay outcomes.
6. Historical updates and discussions of the test procedures should be clearly marked as such and should ideally link to the most recent version of the test procedures to prevent poor referencing for this methodology.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13060544/s1, Supplementary File S1: Table detailing all peer reviewed publications included in the review of the literature and all raw data, and reproducing Table 2.

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Appendix A. Odds Ratio Plots

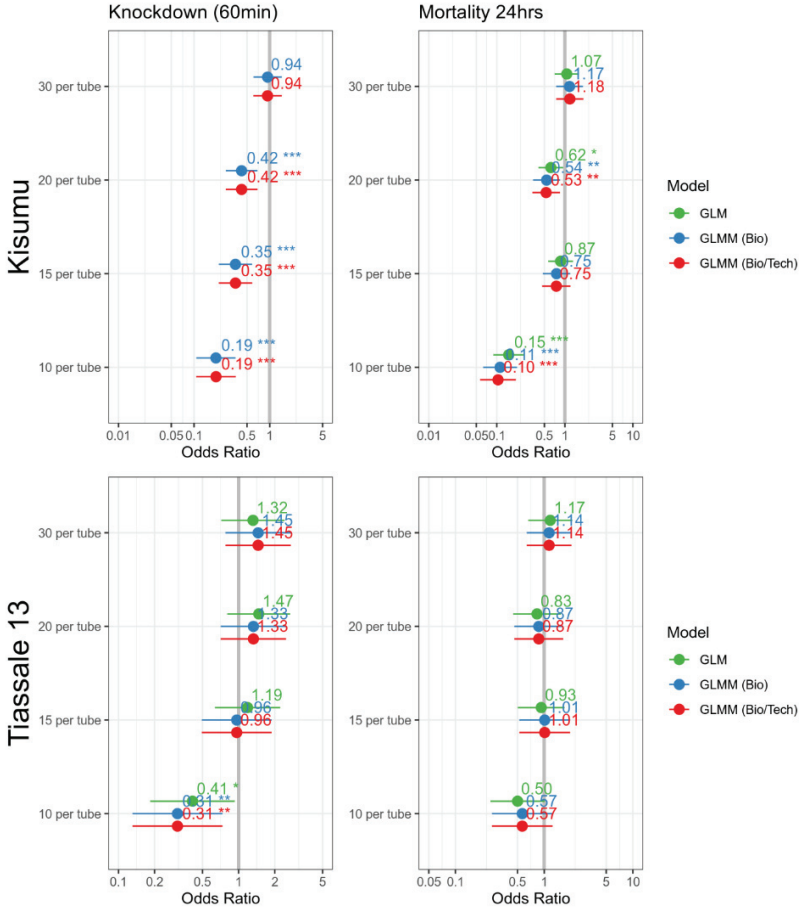

Figure A1. Odds ratio plot for the number of mosquitoes per test unit. The dataset was screened using a binomial GLM, binomial GLMM with random effect for biological replicate, and binomial GLMM with a random effect for biological replicate, and a nested random effect for technical replicate using the glmmTMB package in R. For this analysis, the variable was the age as a factor with 25 mosquitoes used as reference. P-value significance indicated by '*' if ≤0.05, '**' if ≤0.01 and '***' if ≤0.001.

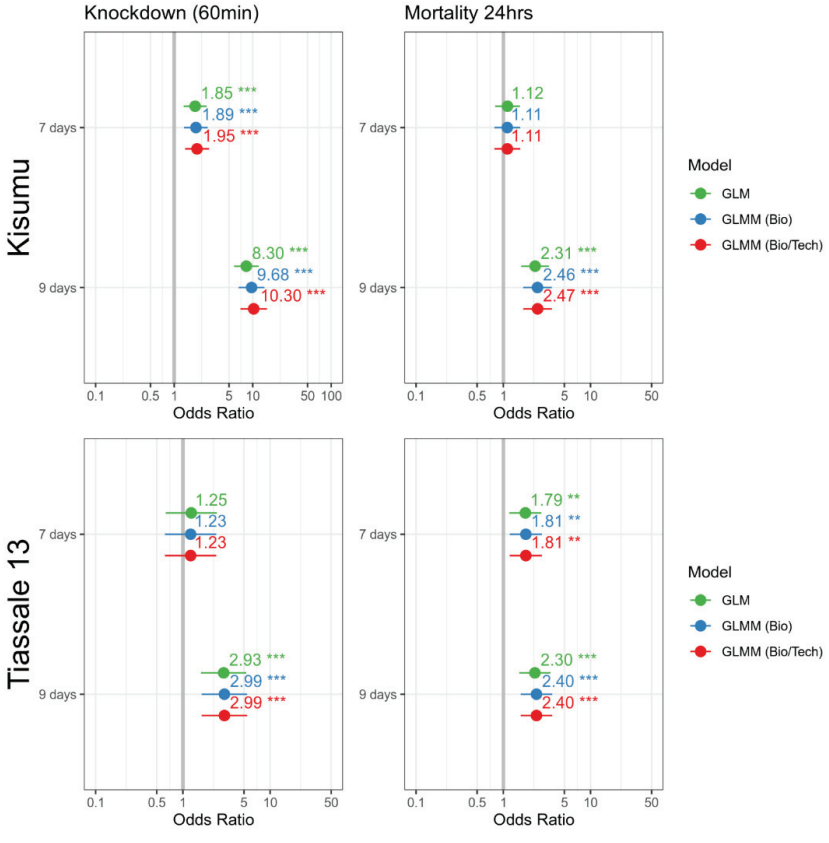

Figure A2. Odds ratio plot for age. The dataset was screened using a binomial GLM, binomial GLMM with random effect for biological replicate, binomial GLMM with a random effect for biological replicate and a nested random effect for technical replicate using the glmmTMB package in R. For this analysis, the variable was the age as a factor with 5 days used as a reference. Age groups are represented by the oldest possible age within that group. P-value significance indicated by '**' if ≤0.01 and '***' if ≤0.001.

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Modified World Health Organization (WHO) Tunnel Test for Higher Throughput Evaluation of Insecticide-Treated Nets (ITNs) Considering the Effect of Alternative Hosts, Exposure Time, and Mosquito Density

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Simple Summary: Membrane feeding assays have been widely used in malaria transmission research and insectary colony maintenance. Here, we investigate whether a membrane feeder can replace animal baits for evaluating insecticide-treated nets (ITNs) bio-efficacy in the World Health Organization (WHO) tunnel test. The effect of (1) alternative baits, (2) exposure time, and (3) mosquito density on the endpoints of mosquito mortality and feeding inhibition or feeding success was investigated. Our results show that similar mortality at 24-h (M24) or 72-h (M72) is estimated using either a membrane feeder or a rabbit bait with an overnight (12 h) exposure. However, the membrane measured higher blood feeding inhibition than the rabbit, likely due to the absence of host cues, notably carbon dioxide. Therefore, the membrane feeder may be used instead of an animal bait to test mortality endpoints in WHO tunnel tests and blood feeding rates need to be improved. Experimental results demonstrated that using 50 or 100 mosquitoes per replicate measure the same for mortality and feeding inhibition endpoints with an animal bait. Therefore, WHO tunnel tests may be run with lower mosquito densities. This will reduce strain on insectaries to produce sufficient mosquitoes to meet the large sample sizes needed for bio-efficacy durability monitoring of chlorfenapyr ITNs that must be evaluated in "free-flying" bioassays.

Abstract: The standard World Health Organization (WHO) tunnel test is a reliable laboratory bioassay used for "free-flying" testing of insecticide-treated nets (ITNs) bio-efficacy where mosquitoes pass through a ITN sample to reach a live animal bait. Multiple parameters (i.e., bait, exposure time, and mosquito density) may affect the outcomes measured in tunnel tests. Therefore, a comparison was conducted of alternative hosts, exposure time, and lower mosquito density against the current gold standard test (100 mosquitoes, animal bait, and 12-h exposure) as outlined in the WHO ITN evaluation guideline. This was done with the aim to make the tunnel test cheaper and with higher throughput to meet the large sample sizes needed for bio-efficacy durability monitoring of chlorfenapyr ITNs that must be evaluated in "free-flying" bioassays. **Methods:** A series of experiments were conducted in the WHO tunnel test to evaluate the impact of the following factors on bio-efficacy endpoints of mosquito mortality at 24-h (M24) and 72-h (M72) and blood-feeding success (BFS): (1) baits (rabbit, membrane, human arm); (2) exposure time in the tunnel (1 h vs. 12 h); and (3) mosquito density (50 vs. 100). Finally, an alternative bioassay using a membrane with 50 mosquitoes (membrane-50) was compared to the gold standard bioassay (rabbit with 100 mosquitoes, rabbit-100). Pyrethroid-resistant *Anopheles arabiensis* and pyrethroid susceptible *Anopheles gambiae* were used to evaluate

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Interceptor® and Interceptor® G2 ITNs. **Results:** Using a human arm as bait gave a very different BFS, which impacted measurements of M24 and M72. The same trends in M24, M72 and BFS were observed for both Interceptor® ITN and Interceptor® G2 unwashed and washed 20 times measured using the gold standard WHO tunnel test (rabbit-100) or rabbit with 50 mosquitoes (rabbit-50). M24, M72 and BFS were not statistically different when either 50 or 100 mosquitoes were used with rabbit bait in the tunnel bioassay for either the susceptible or resistant strains. No systematic difference was observed between rabbit-50 and rabbit-100 in the agreement by the Bland and Altman method (B&A). The mean difference was 4.54% (−22.54–31.62) in BFS and 1.71% (−28.71–32.12) in M72 for rabbit-50 versus rabbit-100. Similar M24, M72 and lower BFS was measured by membrane-50 compared to rabbit-100. No systematic difference was observed in the agreement between membrane-50 and rabbit-100, by B&A. The mean difference was 9.06% (−11.42–29.64) for BSF and −5.44% (−50.3–39.45) for M72. Both membrane-50, rabbit-50 and rabbit-100 predicted the superiority of Interceptor® G2 over Interceptor® ITN for the resistant strain on M72. **Conclusion:** These results demonstrate that WHO tunnel tests using rabbit bait may be run with 50 mosquitoes to increase sample sizes needed for bio-efficacy durability monitoring of ITNs in "free-flying" bioassays. Using a membrane feeder with 50 mosquitoes is a potential replacement for the WHO tunnel bioassay with animal bait if control blood feeding rates can be improved to 50% because blood feeding impacts mosquito survival after exposure to insecticides.

Keywords: WHO tunnel test; insecticide treated nets; ITNs; interceptor; interceptor G2; membrane; human arm; rabbit; bioassay; bio-efficacy; mosquito; *Anopheles*

1. Introduction

Vector control continues to offer effective prevention of mosquito-borne disease globally [1]. Insecticide-treated nets (ITNs) have been an extremely effective control measure [2] because they interrupt malaria transmission in two ways, by reducing mosquito blood-feeding and by killing a proportion of mosquitoes that contact the nets [3,4]. Since 2015, however, malaria control progress has stalled, with the COVID-19 pandemic in 2020 placing additional constraints on malaria control efforts. Despite this, ITNs remain the current cornerstone of global malaria control [1].

To date, all ITNs contain pyrethroid insecticides, which reduce the number of bites that individuals sleeping under them receive even if the nets become old and torn, because pyrethroids inhibit mosquito flight and feeding responses [5,6]. However, mass deployment of pyrethroid ITNs globally has led to widespread pyrethroid resistance with varying mechanisms [7] observed in eighty two countries. To sustain the malaria control gains attributed to ITNs and to assist in reducing malaria by at least 90% by 2030 [8], several ITNs with different insecticide classes in combination with pyrethroids have been developed. These so-called "dual-insecticide ITNs" afford non-neurotoxic modes of action with no cross-resistance (chlorfenapyr), reduced fecundity, and fertility (pyriproxyfen, PPF), or increased susceptibilities to pyrethroids (piperonyl butoxide, PBO) [9–14]. Randomized control trials have demonstrated greater malaria control using dual-active ITNs compared to pyrethroid nets in areas of high pyrethroid resistance, with pyrethroid combined with PBO [15,16], or chlorfenapyr [17]. Operational research has indicated an additional public health benefit of chlorfenapyr [18] and pyriproxyfen [18] in combination with pyrethroid compared to pyrethroid-only ITNs.

New ITN products must demonstrate their continued effectiveness for malaria control up to three years after deployment through biological efficacy testing against mosquito vectors [19,20]. The current World Health Organization (WHO) guidelines for ITN testing outline bioassays that were designed to evaluate pyrethroids with rapid neurotoxic action against exposed mosquitoes, i.e., rapid incapacitation (knockdown), reduction in blood-feeding, and killing within 24 h post-exposure. Chlorfenapyr requires the mosquito to be metabolically or physiologically active (as it is when encountering ITNs during host-

seeking) to metabolize the parent molecule into the potent n-dealkylated form that elicits mosquito mortality [21]. Mosquitoes are more metabolically active at night, when flying, host-seeking or active during their typical circadian rhythms, for which the "free-flying" WHO tunnel test is a more appropriate bioassay [22,23].

The WHO tunnel test is widely used to assess the bio-efficacy of ITNs under laboratory conditions. Despite predicting a similar bio-efficacy of pyrethroid [5] and chlorfenapyr ITNs [22,23] to those measured in gold-standard experimental hut trials, the tunnel test has several limitations. Firstly, the animal baits (rabbit or guinea pig) used are non-preferred hosts for malaria mosquitoes, especially the highly anthropophilic Afrotropical vectors *Anopheles gambiae*, *Anopheles funestus*, and *Anopheles arabiensis* [24,25]. Moreover, the use of animals includes welfare concerns, and it is costly to ensure that animals are well maintained under veterinary supervision. Secondly, the bioassay is conducted overnight for 12–15 h. There is evidence that mosquitoes interact with treated netting within the first 30 min of release [26], thus prolonging exposure time could overestimate outcomes. Thirdly, the current tunnel test uses one hundred mosquitoes per replicate, which is expensive for insectaries to produce. Owing to the significance of blood-feeding in the life cycle of the malaria mosquito, as well as its importance for malaria transmission between human and mosquito hosts, it is an important component of vector control product testing. Different baits could be used to minimize the limitations of using humans or live animal hosts. Membrane feeders have been widely deployed for evaluating topical mosquito repellents [27], transmission-blocking drugs and vaccines [28], and endectocides [29], as well as for mosquito rearing [30–36]. Moreover, the use of an artificial membrane has several advantages, including no animal welfare or ethical concerns, reduced chance of accidental disease transmission, simple logistics, and reproducibility [33,35–37]. Given the significance of host kairomones in encouraging mosquito feeding, worn socks may be added to augment the attractiveness of the membrane to mosquitoes [38].

Multiple parameters including bait [25], mosquito density [39], and duration of exposure to ITNs [40] may affect the outcomes measured in tunnel tests. Therefore, the current paper compared alternative baits, exposure times, and lower mosquito densities against the current gold standard test (100 mosquitoes, animal bait, and 12 h exposure) as outlined in the WHO ITN evaluation guideline [20] in an attempt to simplify the tunnel test to make it cheaper with a higher throughput for the evaluation of large numbers of ITNs as needed for the bio-efficacy durability monitoring of chlorfenapyr ITNs that must be evaluated in "free-flying" bioassays [41].

2. Materials and Methods

2.1. Study Area

Bioassays were performed at the Vector Control Product Testing Unit (VCPTU) facility located at the Bagamoyo branch of Ifakara Health Institute (IHI), Tanzania (6.446° S and 38.901° E).

2.2. Description of Investigational ITNs

Interceptor® is made from 100-denier polyester coated with 200 mg/m^2 alpha-cypermethrin and Interceptor® G2 is made of 100-denier polyester coated with a mixture of 200 mg/m^2 chlorfenapyr and 100 mg/m^2 alpha-cypermethrin. Both net brands are manufactured by BASF, Germany. Safi Net, made of polyester manufactured by A to Z Textile Mills, Tanzania, was used as a negative control to monitor the quality of the bioassay. The study included the following arms: (1) unwashed Interceptor®; (2) Interceptor® washed 20 times; (3) unwashed Interceptor® G2; (4) Interceptor® G2 washed 20 times; (5) negative control–Safi Net. Five samples per net were cut and samples were washed twenty times according to a protocol adapted from the standard WHO washing procedure [20] using 20 g/L palm soap (Jamaa brand). The interval of time used between two washes (i.e., regeneration time) was 1 day for both Interceptor® G2 and Interceptor® ITNs (Table 1).

Table 1. Experimental design.

Experiment	**1**	**2**	**3**		**4**
Factor	**Host/Baits**	**Exposure Time**	**Mosquito Density**		**Replacement of Rabbit**
Comparison	Human or membrane vs. rabbit with 100 mosquitoes	1 h vs. 12 h for human or membrane (within host)	50 vs 100 mosquitoes using rabbit		Rabbit with 100 mosquitoes vs. membrane with 50 mosquitoes
ITNs arms	Interceptor® G2 Unwashed Interceptor® G2 Washed 20× Interceptor® Unwashed Interceptor® Washed 20× Negative control		Interceptor® G2 Unwashed Interceptor® G2 Washed 20× Negative control	Interceptor® Unwashed Interceptor® Washed 20× Negative control	Interceptor® G2 Unwashed Interceptor® G2 Washed 20× Interceptor® Unwashed Interceptor® Washed 20× Negative control
Replicates per arm per comparison	5		15	15	15
Total replicates	75	100	90	90	150
Number of nights	15	10	10	10	16
Mosquitoes exposed	100		100, 50		100, 50
Host/bait	Rabbit, Human, Membrane	Human, Membrane	Rabbit		Rabbit-100, Membrane-50
Exposure time	12 h	12 h 1 h	12 h		12 h
Mosquito species	Anopheles arabiensis		Anopheles arabiensis	Anopheles gambiae	Anopheles arabiensis
Primary Outcomes	Blood feeding success (BFS), 24-h mortality (M24), 72-h mortality (M72)				
Additional Outcome	Blood feeding Inhibition (BFI)				

2.3. Mosquitoes

Pyrethroid-resistant *Anopheles arabiensis* (Kingani strain, established 2017) and pyrethroid susceptible *Anopheles gambiae* (Ifakara strain, established 1996) were used in this study. *An. arabiensis* (Kingani) is metabolic-resistant and expresses the upregulation of cytochrome p450s, with 14% mortality upon exposure to WHO 1x discriminating dose of alpha-cypermethrin that was reversed by piperonyl butoxide (PBO) pre-exposure, reconfirmed before this study was initiated. *An. gambiae* s.s. (Ifakara) is fully susceptible to all insecticide classes at 1x WHO discriminating doses, reconfirmed before this study was initiated. The mosquito colony was maintained according to MR4 Guidelines [36] at 27 ± 2 °C and 40%–100% relative humidity, with an ambient (approximately 12:12) light–dark cycle. The colony was maintained on a Tetramin fish food for larvae, 10% glucose for adults. Females were offered cattle blood in a membrane feeder or were offered a human arm as a blood source. Mosquitoes were 5–8 days old, nulliparous, sugar starved for eight hours, and acclimatized to the test room for an hour before the experiment (Table 1). As VCPTU do not have resistant *An. gambiae* in the colony, we used metabolic resistant *An. arabiensis* instead. Since the bioassay measured contact toxicity, it was deemed that the mechanism for resistance was more critical than the species used for the evaluation.

2.4. The Standard WHO Tunnel Test Procedure

WHO tunnel tests were conducted following WHO guidelines [20] (Figure 1A). The tunnel was divided into two chambers separated by a netting sample that were deliberately holed with 9 small (1 cm) holes through which the mosquitoes had to pass to reach the bait. The bait was placed in the short chamber. In the long section, 100 unfed female mosquitoes aged 5–8 days were released at 19:00 h. The tunnel was covered with a black cloth and left overnight. The following morning, between 07:00 and 09:00 h, mosquitoes were removed from the tunnel using an aspirator. Mosquitoes were scored as alive fed, alive unfed, dead fed, or dead unfed in each chamber and put into a separate paper cup for post exposure mortality monitoring. Mosquitoes were supplied with access to 10% sugar solution *ad libitum* and were then scored for post-exposure delayed mortality at 24-h and 72-h. The experiment and post exposure holding was conducted at a temperature of 27 ± 2 °C and a relative humidity of 80% ± 10. For the experiment to be considered valid, the following thresholds were used: control 24-h mortality ≤10% in all experiments and blood-feeding success ≥50% with experiments using the rabbit bait.

Figure 1. WHO tunnels for comparison of baits: (**A**) Conduct of standard WHO Tunnel with the bait chamber to the left of the picture and mosquitoes being placed into the longer end of the chamber; (**B**) Rabbit—in Experiments 1–4; (**C**) Hemotek® membrane—in Experiment 1 and 4; and (**D**) Human arm—in Experiment 1.

2.5. Bait Used and Preparation

Rabbit: three groups of five healthy rabbits were used. Rabbits were maintained under veterinary supervision. The rabbit was shaved on its back to allow the mosquitoes to feed. The rabbit was gently restrained in a mesh tube that was suspended in the short section of the WHO tunnel throughout the 12-h experiment (Figure 1B). **Membrane feeding:** A Hemotek® membrane feeder (SP-6 System, Hemotek Ltd., Blackburn BB6 7FD, UK) was used. Two membrane feeders were placed on top of the "bait chamber" of each tunnel (Figure 1C). Each feeder reservoir contained 3 mL of cow blood covered by a stretched parafilm membrane and tightened with an o-ring to prevent any leakage. Cow blood was obtained from cattle maintained under veterinary supervision at VCPTU and was stored for up to two weeks at 4–8 °C in heparinized tubes. Socks worn by the investigator (DK)

for 8 h on the day of testing were stretched across the surface of the membrane feeder reservoir to provide host kairomones and increase mosquito attraction to the feeder. The Hemotek® was switched on 10 min before the experiment. The temperature of the feeder was set at 37–39 °C throughout the 12-h experiment. **Human arm:** Five healthy male volunteers conducted arm feeding by inserting their arms into the bait short section of the tunnel (Figure 1D). Before testing, their arms were washed with water. The volunteers were non-smokers and did not drink alcohol or use perfumed lotions during the experimental period. The experimental time for arm feeding was 1 h to allow for standardized evaluation and to minimize volunteer discomfort. Previous work has shown that 30 min of exposure resulted in high blood feeding [35]. To protect human participants, several procedures are routinely undertaken in the laboratory. Anybody who works in the insectary and blood-feeds mosquitoes (including the participants) are screened weekly for malaria parasites using malaria rapid tests (SD bioline). Colony mosquitoes are not kept beyond 10 days, as it takes 12–14 days for mosquitoes to develop sporozoites. Mosquitoes used in the experiments were nulliparous. Therefore, participants were not at risk of malaria infection as a result of the experiments.

2.6. Study Design

Experiments were comparative bioassays with a minimum of 5 replicates per net type, per permutation (Table 1). A total of sixty one experimental nights were run between March 2021 and February 2022. All procedures for preparation, release, collection, and mosquito scoring were performed as per the standard WHO tunnel test procedure [20] (Figure 1A) outlined above with the factors of interest (bait, exposure time, and density) varied (Table 1). The endpoints measured were blood feeding success (BFS) or blood feeding inhibition (BFI), mortality at 24-h (M24), and mortality at 72-h (M72).

2.6.1. Experiment 1: The Impact of Bait/Host

The bio-efficacy of unwashed and 20 times washed Interceptor® G2 and Interceptor® ITNs was tested using 100 pyrethroid-resistant *An. arabiensis* per replicate with membrane, human arm, and rabbit bait (Figure 2A). Mosquitoes were left in the tunnel for 12 h overnight and BFS, M24, and M72 endpoints were evaluated. Five samples for each ITN type and condition (Interceptor® G2 unwashed and 20× washed and Interceptor® unwashed and 20× washed) for each host type were evaluated using five tunnels. One control and four treatments—i.e., one per net type and condition—were conducted each night for 15 nights with each bait (membrane, human, and rabbit) and were evaluated for five nights each. Each bait type was tested on different nights to allow for an independent comparison of each bait in the absence of competing host kairomones.

2.6.2. Experiment 2: The Impact of Exposure Time

The bio-efficacy of unwashed and 20× washed Interceptor® G2 and Interceptor® was tested using 100 pyrethroid-resistant *An. arabiensis* per replicate with either a human arm or membrane bait (Figure 2B). When investigating 1 h exposure, mosquitoes were exposed to ITNs for only 1 h with a human arm or membrane and were then removed from the tunnel and placed in holding cups with access to sugar for 11 h overnight. For the 12-h exposure, the human arm was only available for 1 h, but the mosquitoes were left in the tunnel for the remaining 11 h of the experiment. In the membrane assay, mosquitoes interacted with membrane in the tunnel throughout the 12 h of exposure. In both tests, the BFS, M24, and M72 endpoints were evaluated. Five samples for each ITN type (Interceptor® G2 unwashed and 20× washed and Interceptor® unwashed and 20× washed) plus a negative control were tested using five tunnels. Five replicates per treatment arm for each bait and exposure time were conducted over 10 nights. The 1 h and 12 h of exposure were conducted on the same night for either the membrane or the human arm. The 1 h exposure was performed and then a 12-h exposure was conducted on the same net using a fresh batch of mosquitoes.

Each bait type was tested on different nights to allow for an independent comparison of each bait in the absence of competing host kairomones.

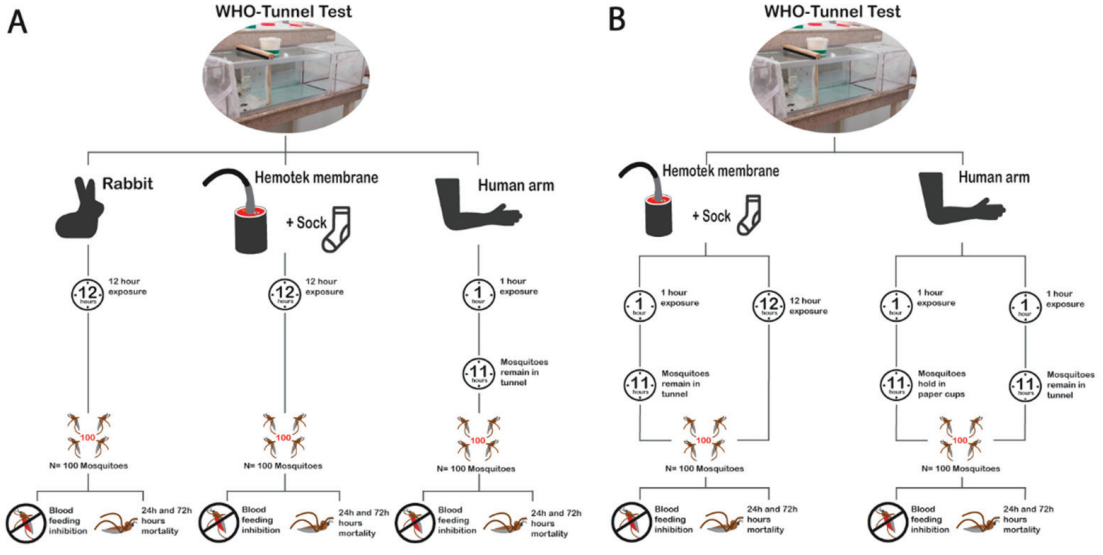

Figure 2. Flow chart of experimental procedure, experiment 1 (**A**—impact of baits) and experiment 2, (**B**—effects of exposure time 12-h vs. 1-h) on WHO tunnel test outcomes.

2.6.3. Experiment 3: Effects of Mosquito Density on the Bio-Efficacy Measurement of Blood-Feeding Inhibition and Mortality at 24-h or 72-h

The effect of mosquito density on bio-efficacy measurements of BFS, M24, and M72 endpoints was evaluated in the WHO tunnel using 50-mosquitoes compared to the standard 100-mosquitoes (Figure 3A). Experiments were conducted following standard procedures with 12 h of exposure and continuous access to a restrained rabbit. For this, two species were used: pyrethroid-resistant *An. arabiensis* tested for the pyrethroid and chlorfenapyr Interceptor® G2 (unwashed or 20× washed) and pyrethroid-susceptible *An. gambiae* for the pyrethroid only Interceptor® ITN (unwashed or 20× washed). A total of seven tunnels (one control, 3 with unwashed, and 3 with washed ITNs) per night were run with 15 replicates conducted per net condition for each mosquito density. Each strain and density (Table 1) were evaluated in a separate 5-night block. This was done to ensure the fitness of mosquitoes used, as the experiments were conducted at a time when the mosquito colony was under pressure from multiple evaluations.

2.6.4. Experiment 4: Possibility to Replace Standard Bait (Rabbit) with the Membrane Assay

To determine whether the rabbit can be replaced with the membrane assay as the bait, the bio-efficacy measurements of BFS, M24, and M72 endpoints were evaluated in the WHO tunnel with 12 h of exposure using 50-membrane and 100-rabbit (gold standard) with resistant *An. arabiensis* mosquitoes (Figure 3B). The same procedure was used for all five arms: a negative control and four treatment arms of Interceptor® unwashed or 20× washed and Interceptor® G2 unwashed or 20× washed (Table 1). For the membrane, a total of 5 tunnels (1 per arm) were run per night, and for the rabbit, 9 tunnels (1 control and 2 replicates per treatment arm) were run per night, with a total 15 replicates per arm for each assay. Different baits were run on separate nights to allow for an independent comparison of each bait in the absence of competing host kairomones.

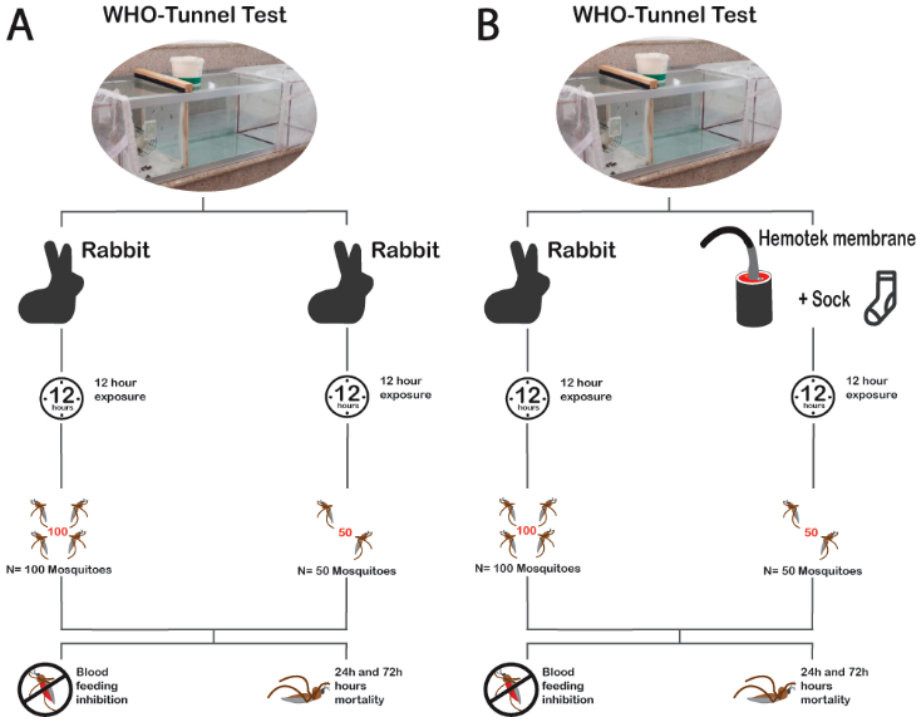

Figure 3. Flow chart of experimental procedure: Experiment 3 (**A**—effects of mosquito density 100 vs. 50) and Experiment 4 (**B**—possibility to replace 100-rabbit bioassay with 50-Hemotek membrane).

2.7. Data Analysis

2.7.1. Sample Size and Power

A sample size calculation for generalized linear mixed effects models (GLMMs) through simulation [42] in R statistical software 3.02 https://www.r-project.org/ (accessed on 23 April 2022) was performed to detect a 10% effect difference between the nets, simulations were performed using an estimated mosquito mortality of 80% for unwashed Interceptor® G2 and 70% for unwashed Interceptor®, and 10% for SafiNet® (deliberately holed). The power estimated was more than 90% based on estimates from previous studies conducted in the same setting: mean mortality of 81.5% for the WHO tunnel test with an assumed daily variation of 0.5 and 15 replicates per arm [23].

2.7.2. Statistical Analysis

Data were collected using standard paper forms and double entered into an Excel spreadsheet, cleaned, and imported into STATA 16.1 (Stata Statistical Software: Release 16. College Station, TX, USA: StataCorp LLC.) for analysis. Descriptive statistics were used for data summarization, whereby mean percentage mortality at 24-h (M24) or 72-h (M72) or blood feeding success (BFS) or blood-feeding inhibition (BFI) with their 95% Confidence Intervals (CI) were calculated. Multivariable mixed logistic regression with a binomial link was conducted with fixed effects for the exposure of interest, adjusting for ITN condition and mosquito species, with day as a random effect to account for daily variability in environmental conditions and mosquito batch variability. Model fit was checked by the testing of model residuals. To estimate the superiority of Interceptor® G2 over Interceptor® with resistant mosquitoes, the same regression was used for comparing superiority measured using the gold standard 100-rabbit to 50-membrane on M72 and

the BFS endpoint. In addition, Bland and Altman [43] methods were used to estimate the agreement in outcomes M24, M72 and BFS measured by assays: (1) membrane vs. rabbit; (2) 100 vs. 50 mosquitoes; and (3) 100-rabbit vs. 50-membrane.

3. Results

3.1. Experimental Validity

In all the bioassays conducted, control M24 was <10% and at M72 was <13%. BFS was ≥50% in both the human arm and the rabbit controls and was <23% in the membrane control (Table 2).

Table 2. Impact of bait on mortality and blood-feeding adjusted for the net condition. The difference in the odds of mosquito mortality at 24-h (M24) or 72-h (M72) and blood feeding success (BFS) for 100 pyrethroid-resistant *Anopheles arabiensis* exposed to Interceptor® and Interceptor® G2 with either a rabbit, human arm or membrane feeder as bait *.

	BFS			M24			M72		
	% (95% CI)	OR (95%CI)	*p*-Value	% (95% CI)	OR (95%CI)	*p*-Value	% (95% CI)	OR (95%CI)	*p*-Value
Control									
Rabbit	64.8 (51.2–78.3)	1		3.8 (0.8-6.8)	1		7.7 (5.1–10.3)	1	
Membrane	22.8 (10.4–35.1)	0.16 (0.14–0.20)	<0.001	6.8 (5.9–7.6)	1.83 (1.22–2.75)	0.004	8.9 (8.3–9.5)	1.16 (0.84–1.59)	0.366
Human arm	74.4 (67.9–80.8)	1.59 (1.25–2.02)	<0.001	6.4 (4.9–7.8)	1.71 (1.05–2.77)	0.030	11.7 (9.0–14.4)	1.58 (1.11–2.26)	0.012
Treatment									
Rabbit	6.6 (2.2–11.0)	1		49.7 (36.4–62.9)	1		66.1 (55.3–76.9)	1	
Membrane	4.6 (1.5–7.7)	0.34 (0.28–0.48)	<0.001	46.5 (35.7–57.3)	0.90 (0.79–1.02)	0.086	67.2 (57.0–77.3)	1.07 (0.93–1.22)	0.352
Human arm	55.9 (49.1–62.7)	9.81 (8.25–11.67)	< 0.001	29.3 (22.1–36.5)	0.42 (0.37–0.48)	<0.001	37.3 (29.7–45.0)	0.31 (0.27–0.35)	<0.001

* Mosquitoes were exposed for 12 h. Data presented are mean proportion (%) with 95% confidence interval (95% CI) and odds ratios (OR) derived from regression analysis with 95% CI adjusted for net type and condition.

3.2. Experiment 1: The Impact of Baits

The bait used affected both the feeding and mortality endpoints measured. The membrane measured a similar mortality and a lower blood feeding success than the rabbit. The human arm measured a lower mortality and higher blood feeding success than the rabbit.

M24 in the intervention arms was not significantly different between the rabbit and membrane (OR: 0.90, 95% CI: 0.79–1.02, $p = 0.086$) and was significantly lower using the human arm (OR: 0.42, 95% CI: 0.37–0.48, $p < 0.001$) compared to the rabbit (Table 2). M72 in the intervention arms was not significantly different between the rabbit and membrane (OR: 1.07, 95% CI: 0.93–1.22, $p = 0.352$) and was significantly lower using the human arm (OR: 0.31, 95% CI: 0.27–0.35, $p < 0.001$) compared to the rabbit (Table 2). Control M24 was higher in the membrane and human arms; but control M72 was higher in the human arm (OR: 1.83, 95% CI: 1.22–2.75, $p = 0.004$) and was not different between rabbit and membrane (OR: 1.16, 95% CI: 0.84–1.59, $p = 0.366$). In the treatment arm, BFS was significantly lower using a membrane (OR: 0.34, 95% CI: 0.28–0.48, $p < 0.001$) and was significantly higher using a human arm (OR: 9.81, 95% CI: 8.25–11.67, $p < 0.001$) compared to the rabbit (Table 2). The same trend was observed in the control arm.

The same trends in mortality and blood feeding inhibition (BFI) were observed for both Interceptor® ITN and Interceptor® G2 (Figure 4). Higher blood feeding resulted in lower mortality (Figure S1), which will explain the lower mortality measured with the human arm, which also had substantially higher BFS. Therefore, the human arm could not be considered for further evaluation. Between the membrane and the rabbit with 100 mosquitoes per replicate, no systematic difference was observed for agreement by Bland and Altman methods (Figure S2). The mean difference was 6% (−10.81–23.01) for BFS and −1.09% (−72.91–70.73) for M72.

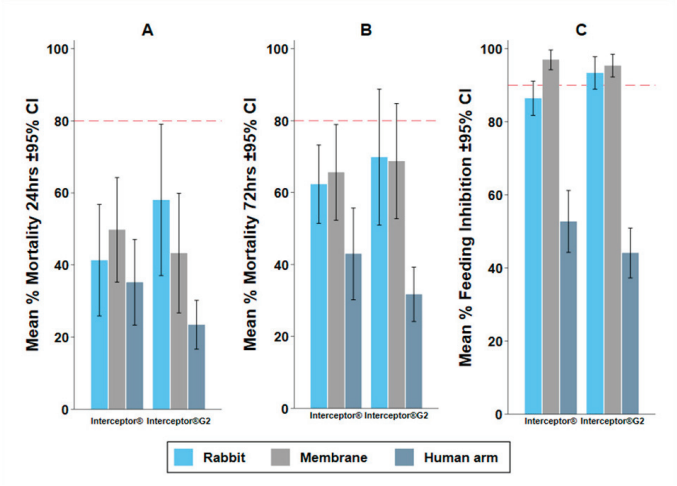

Figure 4. Mean percentage mortality and 95% Confidence Interval (CI) for mortality at (**A**) 24-h (M24), (**B**) 72-h (M72) post exposure and (**C**) blood feeding inhibition (BFI) for Interceptor® and Interceptor® G2 nets with 100 pyrethroid-resistant *Anopheles arabiensis* mosquitoes using rabbit, Hemotek® membrane feeders and human arm as bait in the WHO tunnel bioassay. Red dashed line depicts the WHO minimum bioefficacy criteria of ≥80% M24 and ≥95% BFI.

3.3. Experiment 2: Impact of Exposure Time on Mortality and Blood Feeding

Increasing the time that mosquitoes are left in the tunnel from 1-h to 12-h increased mortality with either the human arm or the membrane (Table 3). With the membrane bait, longer exposure significantly increased both the odds of M72 (OR: 2.30, 95% CI: 2.02–2.62, $p = 0.001$) and the odds of BFS (OR: 1.55, 95% CI: 1.08–2.22, $p = 0.017$). Similarly, in the human arm, the longer exposure significantly increased the odds of M72 (OR: 1.66, 95% CI: 1.45–1.90, $p = 0.001$), while the effect of exposure time on BFS could not be measured since the human arm was only available for one hour (Figure 5). The time that mosquitoes are left in the tunnel overnight is a significant factor in mosquito mortality and should always be recorded and reported.

Table 3. Impact of exposure time on mortality and blood-feeding adjusted for the net condition; The difference in the odds of mosquito mortality at 24-h (M24) or 72-h (M72) and blood-feeding success (BFS)) for 100 pyrethroid-resistant *Anopheles arabiensis* exposed to Interceptor® and Interceptor® G2 with either a human arm or a membrane feeder as bait *.

Assays	BFS			M24			M72		
	% (95% CI)	OR (95% CI)	*p*-Value	% (95% CI)	OR (95% CI)	*p*-Value	% (95% CI)	OR (95% CI)	*p*-Value
Membrane									
1 h-exposure	1.2 (0.1–2.3)	1		24.7 (17.0–32.4)	1		38.9 (26.5–51.2)	1	
12 h-exposure	4.6 (1.5–7.7)	1.55 (1.08–2.22)	0.017	43.3 (25.9–60.6)	1.66 (1.46–1.89)	<0.001	68.8 (52.0–85.5)	2.30 (2.02–2.62)	<0.001
Human arm									
1 h-exposure		NA		20.3 (17.7–22.8)	1		31.1 (26.1–36.1)	1	
12 h-exposure		NA		35.2 (22.7–47.6)	2.26 (1.93–2.64)	<0.001	43.0 (29.6–56.3)	1.66 (1.45–1.90)	<0.001

* Mosquitoes were exposed for either 1 h before being removed from the tunnel and placed in holding cups with access to sugar or left overnight in the tunnel for 12 h. Data presented are a mean proportion (%) with a 95% confidence interval (95% CI) and odds ratios (OR) derived from regression analysis with 95% CI adjusted for net conditions.

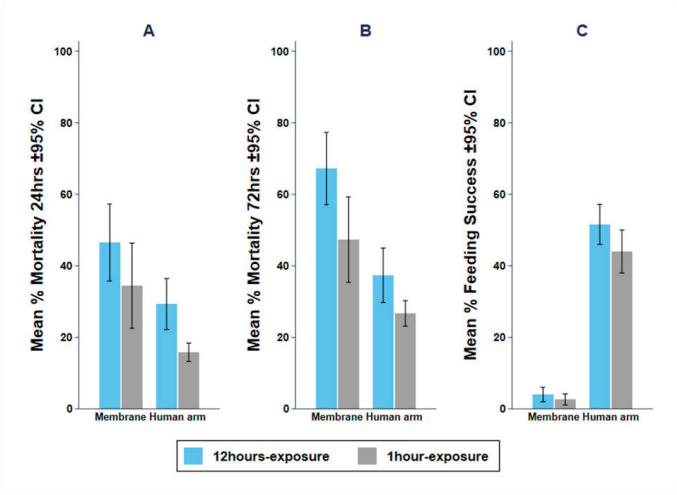

Figure 5. Mean and 95% Confidence Interval (CI) for (**A**) 24-h (M24); (**B**) 72-h (M72); and (**C**) blood feeding success (BFS) with 100 pyrethroid-resistant *Anopheles arabiensis* mosquitoes with 12 h or 1 h exposure time in the WHO tunnel bioassay using Hemotek® membrane or human arm as bait.

3.4. Experiment 3: Effects of Mosquito Density on Tunnel Test Endpoints

M24, M72, and BFS were very similar and were not statistically different when either 50 or 100 mosquitoes were used in the tunnel bioassay with rabbit bait for either the susceptible or resistant strains (Table 4). This was consistent for both Interceptor® and Interceptor® G2, unwashed and washed 20 times (Figure 6). No systematic difference in agreement between methods was observed by Bland and Altman methods (Figure S3). The mean difference was −4.54% (−31.62–22.54) in BFS and 1.71% (−28.71–32.12) in M72. Furthermore, when tested using the pyrethroid-resistant strain, the 50-rabbit bioassay predicted the superiority of Interceptor® G2 to Interceptor®, as did the 100-rabbit (Table 5).

Table 4. Effects of mosquito density on mortality and blood-feeding. The difference in the odds of mosquito mortality at 24 h (M24) or 72 h (M72) and blood feeding success (BFS) for resistant *Anopheles arabiensis* exposed to Interceptor® G2 or susceptible *Anopheles gambiae* to Interceptor® in the gold standard rabbit-100 and 50-rabbit mosquitoes *.

Density	BFS			M24			M72		
	% (95% CI)	OR (95% CI)	*p*-Value	% (95% CI)	OR (95% CI)	*p*-Value	% (95% CI)	OR (95% CI)	*p*-Value
Susceptible *An. gambiae* with Interceptor®									
100 Mosquitoes	5.8 (3.4–8.2)	1		98.3 (97.5–99.1)	1		99.1 (98.6–99.6)	1	
50 Mosquitoes	9.1 (6.6–11.6)	2.35 (0.80–6.92)	0.122	98.4 (97.5–99.3)	1.10 (0.32–3.72)	0.882	99.6 (99.3–99.9)	1.80 (0.43–7.54)	0.421
Resistant *An. arabiensis* with Interceptor® G2									
100 Mosquitoes	12.5 (8.9–16.0)	1		51.8 (41.9–61.7)	1		73.9 (66.7–81.2)	1	
50 Mosquitoes	18.3 (13.3–23.2)	1.54 (0.74–3.22)	0.249	45.1 (40.7–49.6)	0.69 (0.23–2.12)	0.518	70.0 (67.0–73.1)	0.65 (0.25–1.67)	0.375

* Mosquitoes were exposed for 12 h in the tunnel. Data presented are a mean proportion (%) with 95% confidence interval (95% CI) and odds ratios (OR) derived from regression analysis with 95% CI adjusted for net type and condition.

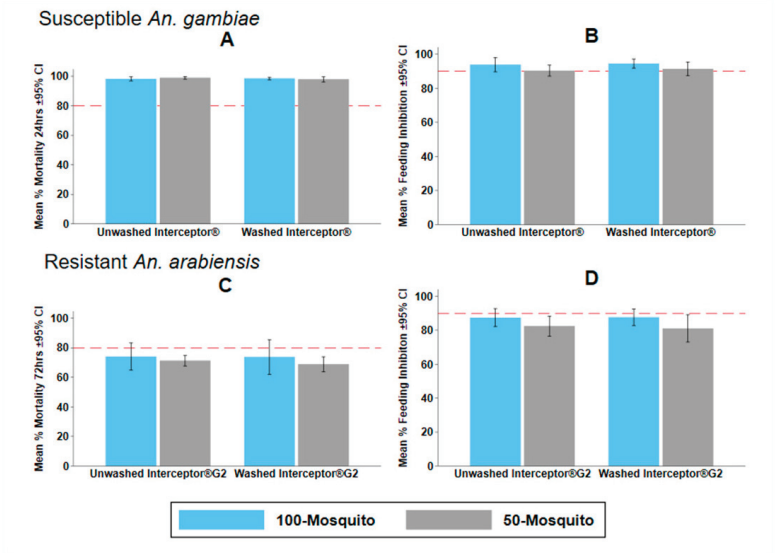

Figure 6. Mean and 95% confidence Interval (CI) for (**A**) 24 h mortality (M24), (**B**) blood feeding inhibition (BFI) of Interceptor® ITN with 100 vs. 50 pyrethroid susceptible *Anopheles gambiae*; (**C**) 72-h mortality (M72); and (**D**) BFI of Interceptor® G2 ITN with 100 vs. 50 pyrethroid-resistant *Anopheles arabiensis* in the WHO tunnel test. Red dashed line depicts WHO minimum bioefficacy thresholds of ≥80% M24 and ≥95% BFI.

Table 5. Superiority of Interceptor® G2 over Interceptor® using 100 versus 50 resistant mosquitoes: The difference in the odds of mosquito at 24 h (M24) and 72 h (M72) and blood feeding success (BFS) for pyrethroid-resistant *Anopheles arabiensis* exposed to Interceptor® G2 and Interceptor® in the gold standard rabbit-100 and 50-rabbit mosquitoes *.

Treatment	100-Rabbit				50-Rabbit			
	BFS		M72		BFS		M72	
	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Overall								
Interceptor®	1		1		1		1	1
Interceptor® G2	1.76 (1.55–1.99)	<0.001	1.23 (1.13–1.33)	<0.001	12.93 (9.63–17.36)	<0.001	1.41 (1.26–1.57)	<0.001
Unwashed								
Interceptor®	1		1		1		1	1
Interceptor® G2	1.64 (1.38–1.95)	<0.001	1.15 (1.02–1.29)	0.018	8.50 (5.95–12.15)	<0.001	1.83 (1.56–2.14)	<0.001
Washed 20×								
Interceptor®	1		1		1		1	1
Interceptor® G2	1.90 (1.58–2.27)	<0.001	1.31 (1.17–1.47)	<0.001	24.34 (14.16–41.85)	<0.001	1.07 (0.85–1.34)	0.432

* Mosquitoes were exposed for 12 h in the tunnel. Data presented are a mean proportion (%) with a 95% confidence interval (95% CI) as well as odds ratios (OR) derived from regression analysis with 95% CI, adjusted for net conditions.

However, when considering the superiority of Interceptor® and Interceptor® G2, the lower mosquito density (50) resulted in a higher BFS in the Interceptor® G2 arm (Table 5). This indicates that mosquitoes at a high density are either interacting with each other to disturb each other from feeding, or discomfort from high biting rates is making the host more defensive. This increased blood feeding success is likely translating into the lower odds of mortality observed for washed Interceptor® G2 relative to Interceptor® using 50 mosquitoes (OR: 1.07, 95% CI: 0.85–1.34, $p = 0.579$) compared to 100 mosquitoes (OR: 1.31, 95% CI: 1.12–1.54, $p = 0.001$) (Table 5). This observation underlines the importance of consistent control blood feeding success on mortality estimates from the WHO tunnel test and this should always be recorded and reported.

3.5. Experiment 4: Possibility to Replace Standard Bait with the Membrane Feeding

The membrane assay with 50 mosquitoes (membrane-50) did not measure statistically different M24 or M72 compared to the rabbit with 100 mosquitoes (rabbit-100) (Table 6) when testing pyrethroid only Interceptor® or Interceptor® G2 against pyrethroid-resistant *An. arabiensis*. Again, BFS was different, with a far higher BFS in the rabbit-100 assay than in the membrane 50-assay.

Table 6. Comparison of the membrane assay to the gold standard with rabbit assay. The difference in the odds of mosquito mortality at 24-h (M24) and 72-h (M72) and blood feeding success (BFS) for resistant *Anopheles arabiensis* was measured between the gold standard rabbit assay with 100 mosquitoes and the membrane assay with 50 mosquitoes *.

Assay	BFS		*p*-Value	M24		*p*-Value	M72		*p*-Value
	% (95% CI)	OR (95% CI)		% (95% CI)	OR (95% CI)		% (95% CI)	OR (95% CI)	
Interceptor®									
100 Rabbit	7.9 (4.1–11.8)	1		56.4 (45.3–67.6)	1		69.4 (60.0–78.8)	1	
50 Membrane	1.2 (0.8–1.7)	0.19 (0.08–0.45)	<0.001	52.5 (45.6–59.4)	0.39 (0.10–1.61)	0.195	73.0 (66.9–79.0)	0.54 (0.14–2.06)	0.370
Interceptor® G2									
100 Rabbit	12.5 (9.0–16.0)	1		51.8 (42.0–61.7)	1		73.9 (66.7–81.1)	1	
50 Membrane	2.3 (1.3–3.2)	0.17 (0.09–0.30)	<0.001	56.4 (49.8–63.1)	1.10 (0.51–2.36)	0.814	83.0 (79.1–86.9)	1.50 (0.75–2.98)	0.251

* Mosquitoes were exposed for 12 h in the tunnel. Data presented are a mean proportion (%) with a 95% confidence interval (95% CI) as well as odds ratios (OR) derived from regression analysis with a 95% CI adjusted for net type.

However, when used for predicting the difference in bio-efficacy between Interceptor® and Interceptor® G2, both assays were measured in the same way (Figure 7) and both predicted superior odds of M72 for Interceptor® G2 (100-rabbit OR: 1.23 (95% CI: 1.10–1.38), $p < 0.0001$; 50-membrane 1.79 (95% CI: 1.50–2.14) $p < 0.0001$) and inferior reduction in blood feeding (100-rabbit OR: 1.76 (95% CI: 1.47–2.10), $p < 0.0001$; 50-membrane 1.87 (95% CI: 1.05–3.33) $p = 0.033$) with Interceptor® G2 relative to Interceptor® (Table 7). No systematic difference was observed in agreement for membrane-50 and rabbit-100 by Bland and Altman methods, with a mean difference (and limits of agreement) of 9.06 % (−11.42–29.54) on BFS and −5.43 % (−50.3–39.45) on M72 (Figure S4).

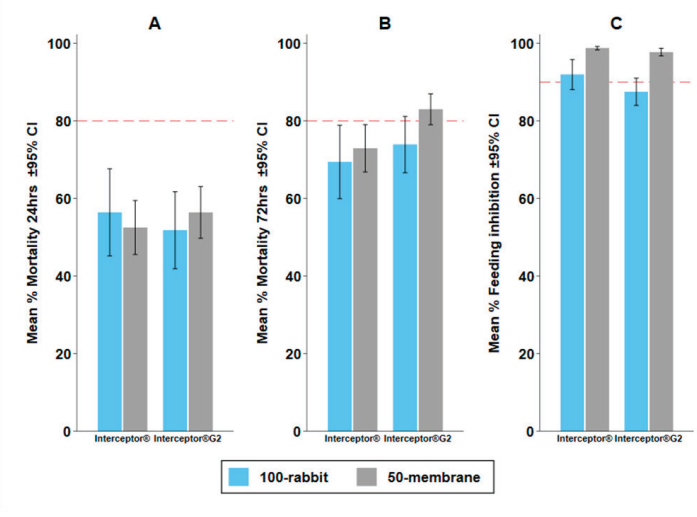

Figure 7. Mean percentage mortality and 95% confidence interval (CI) for (**A**) 24-h (M24); (**B**) 72-h (M72); and (**C**) blood feeding inhibition (BFI) for Interceptor® and Interceptor® G2 nets against pyrethroid-resistant *Anopheles arabiensis* with 100-rabbit (rabbit bait and density of 100 mosquitoes) and 50-membrane (Hemotek® membrane bait and density of 50 mosquitoes) in the WHO tunnel test. The red dashed line depicts the WHO minimum bio-efficacy thresholds of ≥80% M24 and ≥95% BFI.

Table 7. Superiority of Interceptor® G2 over Interceptor® was estimated by comparing the membrane assay to the gold standard assay with pyrethroid-resistant mosquitoes. The difference in the odds of mosquito at 72-h (M72) and blood feeding success (BFS) for resistant *Anopheles arabiensis* measuring the superiority of Interceptor® G2 and Interceptor® with the gold standard with 100-rabbit compared to 50-membrane bioassays *.

Treatment	100-Rabbit				50-Membrane			
	BFS		M72		BFS		M72	
	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value	OR (95% CI)	*p*-Value
Overall								
Interceptor®	1		1		1		1	
Interceptor® G2	1.76 (1.47–2.10)	<0.001	1.23 (1.10–1.38)	<0.001	1.87 (1.05–3.33)	0.033	1.79 (1.50–2.14)	<0.001
Unwashed								
Interceptor®	1		1		1		1	
Interceptor® G2	1.64 (1.28–2.09)	<0.001	1.15 (0.98–1.35)	0.094	2.34 (1.11–4.93)	0.025	1.81 (1.43–2.29)	<0.001
Washed 20×								
Interceptor®	1		1		1		1	
Interceptor® G2	1.90 (1.47–2.45)	<0.001	1.31 (1.12–1.54)	0.001	1.26 (0.49–3.20)	0.634	1.82 (1.39–2.37)	<0.001

* For the gold standard, 100-mosquitoes with rabbit and 50-mosquito with 2 Hemotek® membrane feeders augmented with worn socks were used in the WHO tunnel bioassay, adjusted for net type and condition.

4. Discussion

The current tunnel test uses one sample of ITN with 100 mosquitoes as the unit of replication and based on the current work, it is proposed that a larger number of nets or two samples per ITN can be tested using 50 mosquitoes per replicate to improve laboratory throughput. Biological durability monitoring requires large sample sizes, as nets are exposed to highly variable use patterns [44–48] and environmental conditions [49,50] that result in a high degree of heterogeneity between individual nets. The goal of biological durability monitoring is the precise estimation of the biological efficacy of a population of ITNs. As the ITN is the unit of replication, greater precision is obtained by evaluating larger numbers of ITNs.

The current experiment confirmed that using the Hemotek® membrane feeding system as a blood source, together with a worn sock emitting human odor with a replicate size of 50 mosquitoes, a similar mortality and feeding inhibition as the standard WHO tunnel bioassay with rabbit and a replicate size of 100 mosquitoes for both pyrethroid and mixture pyrethroid and chlorfenapyr ITNs is estimated. Our results suggest that a membrane bioassay can evaluate the difference between ITNs because the membrane assay estimates the superiority of Interceptor® G2 over Interceptor® on the M72 outcome using metabolic-resistant mosquitoes, which was also measured by the gold standard rabbit-100 assay and has been consistently seen in other studies in the WHO tunnel, I-ACT, and experimental hut [23]. It was also able to predict the superior blood feeding inhibition of Interceptor®, which has a higher concentration of the pyrethroid alpha-cypermethrin (200 mg/m^2 alpha-cypermethrin in Interceptor® and 100 mg/m^2 alpha-cypermethrin in Interceptor® G2). Being able to test differences between products is the goal of durability monitoring bioassays that track the bio-efficacy of ITNs over time (biological durability) and compare them to unwashed positive controls [41].

Having a reliable bioassay that can be conducted routinely without animal welfare concerns will be extremely useful. The data generated by the current work are promising and further work is planned to improve mosquito feeding success on the membrane as it was seen that differences in blood feeding success do impact on the mortality estimates. While this did not impact on the predictions of superiority, and therefore mortality can still be compared to an unwashed positive control net, if thresholds are used, i.e., the proportion of nets that meet WHO bio-efficacy criteria, then this might affect the interpretation of the bioassay results. It is recommended that the results are replicated in additional laboratories, since having an assay that can accurately predict the differences between net samples in multiple laboratories with several pyrethroid-resistant mosquito strains and that can predict the results of experimental hut studies is optimal. Data from the experiments

demonstrated that several factors influenced the mean mortality and feeding inhibition estimated in WHO tunnel tests [22,51,52].

4.1. Impact of the Bait

The use of different baits had an enormous influence on the bioassays. By using a human arm as bait, feeding inhibition was substantially lower compared to membrane or rabbit baits [53]. This has also been seen in early versions of the tunnel test [51]. This preference for the human arm is unsurprising, since the colony used in the experiments is anthropophilic. Therefore, although it is more representative of end-user conditions, the use of a human is not recommended for ITN evaluation, because the results were not comparable to those of the rabbit bioassay that was shown to predict the results of experimental hut trials in this setting [23] and elsewhere [9,22,52]. Study findings using *An. arabiensis* mosquitoes were consistent with the existing literature on vector host preference [25,54], confirming that mosquitoes are most attracted to humans as bait, followed by rabbits, and were least attracted to the membrane. Lower attraction in assays using the Hemotek® membrane system and rabbits reduces the number of mosquitoes passing the ITN tested, resulting in higher feeding inhibition compared to when the human arm was used as bait. Several other studies have shown that host-seeking *An. arabiensis* are more attracted to humans than to live animals [24,25]. The lower attraction and consequent higher feeding inhibition when using a membrane is likely due to the absence of carbon dioxide (CO_2) that increases mosquito responses to kairomones [55] and the small size of the membrane feeder's surface, which reduces the amount of heat and moisture available, which are both important short-range attractants to mosquitoes [56–58]. The validity of the experiment relies on the negative control feeding success of (>50%) for rabbits. In this assay, with the membrane, augmentation with socks that contained human kairomones improved the attraction of the membrane to mosquitoes [59]. However, it was not possible to use the same threshold value for feeding success with the less attractive membrane. For this reason, further work is needed to optimize the attraction of the membranes for use in the WHO tunnel test. Further improvements to the attractiveness of the membrane could be achieved by making a larger surface area available [60,61] and the addition of 2-butanone [62] or CO_2 [63] to augment mosquito response to kairomones until 50% feeding success in the negative control is consistently achieved.

4.2. Impact of Exposure Time

Exposure time was important with 12 h exposure, increasing both mortality and feeding success, indicating that the mosquitoes make repeated contact with the ITN sample overnight. Consistently, prolonged exposure (12 h) increased the efficacy of insecticide and host-seeking activities compared to 1 h exposure, resulting in increased mortality because of a higher dose of insecticide picked up by the mosquitoes when resting, bouncing, and passing the ITNs repeatedly. This is also likely in experimental huts and in the community where ITNs are in use. Therefore, the use of a 12 h overnight exposure is recommended. For insecticides that require the mosquitoes to be metabolically active, such as chlorfenapyr, prolonging exposure to 12 h allows the conversion of parent molecules into active forms, because of mosquitoes' metabolic activity when flying in the tunnel. Interestingly, results show that with either the pyrethroid only Interceptor® or the pyrethroid-chlorfenapyr Interceptor® G2 ITNs higher mortality was observed among unfed mosquitoes. Therefore, the results of this study underline the WHO recommendation that feeding success should always be reported when conducting WHO tunnel tests, as low feeding rates will affect the interpretation of results.

4.3. Effects of Mosquito Density

It was observed that the use of 50 or 100 mosquitoes per testing sample with the rabbit bait did not significantly alter the mortality and blood feeding success measured with either resistant *An. arabiensis* or susceptible *An. gambiae* for the pyrethroid only net or the

mixture ITNs. These results suggest that fewer mosquitoes can be used in WHO tunnel bioassays and still correctly measure the efficacy of ITNs. As would be expected, with 50 mosquitoes there is a slight increase in blood feeding success and a consequent slight decrease in mortality compared to assays using 100 mosquitoes. Higher feeding success at a lower density is likely due to less competition between mosquitoes on the membrane during host-seeking [35], which may also reduce the host defensiveness of the rabbit [64,65]. Increasing the number of mosquitoes in the tunnel may lead to density-dependent mortality effects of crowding as mosquitoes can disturb each other when at a high density [66]. Our results suggest that regardless of the insecticides on the ITNs tested, mortality was higher among unfed mosquitoes, revealing an impact of blood feeding on increased mosquito resilience to insecticides after a blood meal. A similar study on the effects of bites through permethrin nets shows that successfully fed mosquitoes survive longer than unfed ones [67]. This has been reported for chlorfenapyr, where observed mortality was lower among blood-fed mosquitoes compared to those who were unfed [10]. Blood feeding elevates detoxifying enzymes (glutathione, monooxygenase), which then assist in the detoxification of insecticides [68], although this did not translate into substantially lower bio-efficacy with Interceptor® G2 as upregulation of metabolization converts the parent molecule into the potent n-dealkylated form that elicits increased mosquito mortality [21]. It is also important to report control blood feeding success because unfit colony mosquitoes are less likely to fly and feed, which reduces the likelihood that the mosquitoes contact treated nets [67], nullifying the bioassay.

4.4. Study Limitations

The study has several limitations which should be addressed in subsequent work. Firstly, experiments were conducted in a single testing facility. A comparison of the alternative method in multiple laboratories is desirable to ensure the reproducibility of the methods with other mosquito strains. The low feeding success with the membrane technique needs to be overcome, as clearly feeding success impacts mosquito mortality. Ideally, the membrane bioassay will be improved to consistently measure 50% mosquito feeding success at multiple testing facilities. Additionally, two different ITN products from the same manufacturer were used. It could be argued that the evaluation of dual AI nets of pyrethroids with PBO and PPF would also be as relevant, although these are best measured using WHO cone tests as they do not require "free-flying" bioassays for evaluation. Similar experiments conducted by other facilities are recommended to generate further evidence of the range of values and precision of the estimates of mortality and blood feeding inhibition using the 50-rabbit and 50-membrane technique.

5. Conclusions

Here, it was demonstrated that using 50 or 100 mosquitoes with the rabbit gives similar results with no systematic bias for both pyrethroid and pyrethroid-chlorfenapyr ITNs. The lower density can be used for the WHO tunnel test when testing pyrethroid Interceptor® and pyrethroid-chlorfenapyr Interceptor® G2. Reducing the number of mosquitoes per test decreases its cost and allows a larger number of net samples to be tested at a time. Larger sample sizes will give greater precision when estimating ITN efficacy since the unit of replication in ITNs testing is the bioassay (cone, tunnel, I-ACT, experimental hut) and not the mosquito within that assay. Furthermore, we provide the first evidence that membrane feeding systems can be used as an alternative to rabbit bait in WHO tunnel assays. Membrane assay shows an excellent comparison to the gold-standard WHO tunnel test on both the mortality and feeding success endpoint for the ITNs tested, although control feeding success is lower due to the lower attraction of the membrane to host-seeking mosquitoes. Using membrane feeding systems instead of rabbits or other animals in WHO tunnel assays resolves the ethical issues concerning animal welfare and makes the tests simpler to perform. Further work to improve the feeding success of the membrane

feeding system as a replacement for rabbits in the WHO tunnel test is needed, as mosquito feeding success impacts insecticide induced mortality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13070562/s1, Figure S1: Mean percentage mortality at (A) 24-h and (B) 72-h with rabbit-100, (C) at 24-h and (D) 72-h with membrane-50 of blood fed and unfed resistant *Anopheles arabiensis* in the WHO tunnel test. The Red dashed line depicts (the WHO mortality threshold $\geq$ 80%). Figure S2: Bland and Altman plot of (A) blood feeding success (BFS) and (B) 72-h mortality (M72) for Interceptor® and Interceptor® G2 with rabbit or membrane bait against 100-pyrethroid resistant *Anopheles arabiensis* in the WHO tunnel test with a 12 h exposure time. The average value for both methods is plotted on the *x*-axis and the mean difference between methods on the *y*-axis. The solid line in the middle shows the mean difference with a 95% confidence interval of the mean difference represented by the dashed lines. Figure S3: Bland and Altman plot of A-blood feeding success (BFS) and B-72-h mortality (M72) for Interceptor® with susceptible *Anopheles gambiae* and Interceptor® G2 with resistant *Anopheles arabiensis* using rabbit bait and a density of either 100 or 50 mosquitoes in the WHO tunnel test with a 12-h exposure time. The average value for both densities is plotted on the *x*-axis and the mean difference between densities on the *y*-axis. The solid line in the middle shows the mean difference with a 95% confidence interval of the mean difference represented by the dashed lines. Figure S4: Bland and Altman plot of A-blood feeding success (BFS) and B-72-h mortality (M72) for Interceptor® with susceptible *Anopheles gambiae* and Interceptor® G2 with resistant Anopheles arabiensis using rabbit bait and a density of either 100 mosquitoes or membrane bait and a density of 50 mosquitoes in the WHO tunnel test with a 12-h exposure time. The average value for both densities is plotted on the *x*-axis and the mean difference between densities on the *y*-axis. The solid line in the middle shows the mean difference with a 95% confidence interval of the mean difference represented by the dashed lines. Table S1: Mean percentage mortality and 95% confidence interval (95% CI) for the negative control, Interceptor® G2, and Interceptor® at 24 h post exposure (M24) and mortality at 72 h post exposure (M72) and blood feeding success (BFS) or blood feeding inhibition (BFI) of resistant *Anopheles arabiensis* with 12 h of exposure time for rabbit, membrane and human arm and 1 h exposure time for in membrane and human arm in the WHO tunnel test. The negative control thresholds for the WHO tunnel test are blood feeding success $\geq$ 50% and M24 $\leq$ 10%. Table S2: Mean percentage mortality and 95% confidence interval (95% CI) for the negative control, susceptible *Anopheles gambiae* with Interceptor® resistant *Anopheles arabiensis* with Interceptor® G2 at 24 h post exposure (M24) and mortality at 72 h post exposure (M72) and blood feeding success (BFS) or blood feeding inhibition (BFI) with the density of 50 or 100 mosquitoes in the WHO tunnel test. The negative control thresholds for WHO tunnel test are blood feeding success $\geq$ 50% and M24 $\leq$ 10%. Table S3: Mean percentage mortality and 95% confidence interval (95% CI) for the negative control, resistant *Anopheles arabiensis* with Interceptor ® or Interceptor® G2 at 24 h post exposure (M24) and mortality at 72 h post exposure (M72) and blood feeding success (BFS) or blood feeding inhibition (BFI) with rabbit bait and a density of 100 mosquitoes (rabbit-100) or membrane bait with 50 mosquitoes (membrane-50) in the WHO tunnel test. The negative control thresholds for WHO tunnel test are blood feeding success $\geq$ 50% and M24 $\leq$ 10%.

Author Contributions: The conceived and design of the experiment study was done by D.S.K., S.J.M. The experiment was performed by D.S.K. Data analysis was conducted by D.S.K., S.J.M. and O.G.O., D.S.K. wrote the manuscript. L.H. drew the diagrams. The manuscript was critically revised by S.J.M., E.M., O.G.O., and L.H. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data set for this study is available on reasonable request from Vector Control Product and Testing Unit of Ifakara Health Institute.

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Conflicts of Interest: D.S.K., S.G.M. and L.H., have no competing interests. S.J.M., O.G.O. and E.M. conduct product evaluations for several vector control product manufacturers including B.A.S.F. that manufacture the ITNs tested. B.A.S.F. was not involved in the work or the decision to publish.

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Enhancing the Quality of Spray Application in IRS: Evaluation of the Micron Track Sprayer

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Simple Summary: A key tool in the fight against mosquitoes, which transmit malaria, is the application of insecticidal indoor residual spray (IRS) to the internal walls of buildings where mosquitoes alight and rest to digest their blood-meals. When evaluating the effectiveness of IRS formulations for killing mosquitoes when applied to a wall, it is important that the insecticide is applied evenly at the target dose. Traditionally, IRS is applied using a hand-held pump, but this study showed that an automated track sprayer delivered the desired dose to wall surfaces more accurately and more evenly. This was first shown using a fluorescent tracer to measure spray deposit on the wall of a laboratory, and then by spraying different IRS formulations onto the walls of an experimental hut.

Abstract: Indoor residual spraying (IRS) has changed little since its introduction in the 1940s. Manual spraying is still prone to variation in insecticide dose. To improve the application of IRS in experimental hut trials, an automated track sprayer was developed, which regulates the speed of application and the distance of the nozzle from the wall, two key sources of variation. The automated track sprayer was compared to manual spraying, firstly using fluorescein solution in controlled indoor settings, and secondly in experimental huts in Tanzania using several IRS products. Manual spraying produced greater variation with both fluorescein and insecticide applications. Both manual and automated spray methods under-dosed the actual dose sprayed compared to the target dose. Overall, the track sprayer treats surfaces more consistently, offering a potential improvement over manual spraying for experimental hut evaluation of new IRS formulations.

Keywords: IRS; application technology; broflanilide; clothianidin; deltamethrin; pirimiphos-methyl

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1. Introduction

Indoor residual spraying (IRS) is a cornerstone of malaria vector control. It is typically conducted manually by spray operators using compression sprayers, a method that has seen little change since its introduction in the 1940s. Equipment specifications for IRS were first published in 1964 and described a hand-operated cylindrical tank with a hose, lance, and a flat-fan nozzle [1]. Specifications remained relatively unchanged until the addition of a control flow valve (CFV). Regulating the flow of insecticide through the nozzle with a CFV meant that, despite decreasing pressure in the tank while spraying, the emitted spray volume stayed constant. The introduction of CFVs resulted in a recommended application rate of 30 mL/m^2, rather than the original 40 mL/m^2, reducing the volume (and thus

weight) of water needed to spray a surface area. A second change was the shift from stainless steel nozzles to a more durable ceramic nozzle, reducing the risk of inconsistent spray due to wear on the nozzle [2].

Although these innovations in spray equipment have resulted in more accurate IRS applications, the spray technique itself has remained similar to that in early spray campaigns [3]. In control campaigns, manual spraying is prone to variation in the dose applied due to variation in competence and skills, and lapses in concentration between operators. Even in experimental hut trials, human error by individual spray operators can result in large differences in insecticide application rates between walls within a house and between positions on a single wall. Both overdosing and underdosing of IRS products have been reported during the conduct of experimental hut [4–7], highlighting the challenges in spraying IRS products accurately and consistently. Overdosing of IRS products can lead to higher than anticipated costs and potential safety concerns, while underdosing can result in a shorter residual half-life and development of mosquito resistance due to exposure to sublethal doses [8,9]. Consistency in the speed of application and the distance of the sprayer nozzle from the surface are critical to applying the correct dose of insecticide to walls, ceilings, and other sprayable surfaces.

High-quality training and supervision of spray operators, plus good maintenance and calibration of spray equipment, can contribute to the accurate application of IRS products in experimental hut studies (and in IRS spray campaigns). Accurate quality control of spray application, however, remains challenging and relies on complicated, timely, and expensive technology such as HPLC analysis of sprayed filter papers, or methods that are relatively insensitive to variations in dose such as cone assays. Variation in insecticide delivery has impacted the effectiveness of spray campaigns or outcome of regulatory trials. High accuracy in the measuring of spraying is particularly important in experimental hut trials evaluating different dosages of IRS products [10–15], as these trials need to inform development decisions on the most appropriate application rate for novel products prior to regulatory evaluations and subsequent market launch. To improve the consistency of the application of IRS products, the automated mechanical track sprayer was developed by Micron Sprayers Ltd., with support and funding via the Innovative Vector Control Consortium (IVCC). The track sprayer was designed specifically for experimental use, aiming to improve the quality of insecticide application in semi-field experimental hut studies.

This comparative study was conducted to evaluate whether the application of IRS products by mechanical track sprayer gives less variation in spray application rate than conventional manual spraying. The comparison was made during two experimental phases: the first phase, conducted in the laboratory at the Micron Centre in Herefordshire, England, used fluorescein diluted in water; and the second phase, under semi-field conditions at the Kilimanjaro Christian Medical University College (KCMUCo, Moshi, northern Tanzania) used IRS products. The proof-of-concept laboratory phase allowed for a high-throughput and low-cost comparison of both spray methods, whereas the semi-field phase provided the opportunity to test with insecticidal products under more realistic experimental conditions. For both phases, the manual spraying was carried out by an experienced spray operator and the mechanical spraying was conducted using the automated track sprayer.

2. Materials and Methods

2.1. Spray Methodology

The Micron Track Sprayer (Micron Sprayers Ltd., Bromyard, UK) consists of a conveyer belt along which a spray head with nozzle can move vertically up and down (Figure 1). The speed of the nozzle is adjustable using an electronic hand-held controller, and power is provided by a rechargeable battery pack. The spray head is connected to a pressurized spray tank of the Micron compression sprayer. Extendable arms at the top and bottom of the track sprayer were set 45 cm from the wall surface, and the equipment was levelled

horizontally using a standard spirit level. The travelling speed was set to 0.45 m/s, which corresponds to 1 m sprayed every 2.2 s as per WHO guidelines [2].

Figure 1. A schematic overview of the sprayer adapted from the Micron product manual is shown on the (**left**). A photo showing the set-up of track sprayer in the lab (**centre left**) and in an experimental hut (**centre right**) is shown, and a schematic overview of the filter paper positions on plywood panels, with values in centimetres, is shown on the (**right**).

The Micron track sprayer was compared to manual spraying, performed as detailed in WHO guidelines [2,16]. Before manual spraying, the spray operators were extensively trained using standard operating procedures (SOPs) based on the WHO guidelines which details lance speed and angle, distance from the wall, and speed of movement vertically up and down the walls during application. Spray tanks were calibrated and maintained according to good laboratory practice (GLP) standards. Sprayers were calibrated and deemed acceptable when spraying 550 mL $\pm$ 10% per minute. Spraying was carried out at an application rate of 30 mL/m^2 using a 1.5 bar CFV. To ensure comparability between the two spraying application methods, the same spray tank, insecticide solution, 8002E flat fan nozzle, and CFV were used for both manual spraying and automated track spraying.

For both phases of the comparison, a similar protocol was used; minor differences between the round with fluorescein and the round with insecticides are detailed below.

2.2. Spraying of Fluorescein

The first phase of spraying was performed using 0.1% w/v fluorescein sodium salt diluted in water. A 3.55 m $\times$ 2.00 m tiled wall surface was marked up to accommodate five 75 cm spray swaths (70 cm spray + 5 cm overlap). Each set of five swaths constituted one replicate test. Filter papers (10 cm diameter, Whatman No.5) were held in place in Petri dish lids using a plastic ring; the lids were attached to the wall surface using self-adhesive Velcro strips in a grid pattern with three horizontal positions and five vertical positions per swath (see Figure 1). Vertical positions were located at the following heights: 1.80 m (high), 1.40 m (mid-high), 1.00 m (centre), 0.60 m (mid-low), 0.20 m (low). Horizontal positions were at 0.2 m (left), 0.375 m (centre), and 0.55 m (right) from the left edge of the swath. Six replicates using the track sprayer and five replicates spraying manually were performed using the fluorescein water solution. In total, 1045 filter papers were analysed. Track spraying was performed using a Micron CS10 compression sprayer tank. The target spray rate was 2 metres in five seconds; a metronome app was used to assist the manual spray person to follow an even spray rhythm. The spray time and direction (upwards or downwards) was recorded for each swath.

2.3. Spraying IRS Products

Three IRS products containing different active ingredients were sprayed in experimental huts: broflanilide (VECTRON™ T500, Mitsui Chemical Agro Inc., Tokyo, Japan, batch no 18I-3671), pirimiphos-methyl (Actellic® 300CS, Syngenta, Basel, Switzerland, batch no BSN9A2383), and a deltamethrin + clothianidin combination product (Fludora Fusion®, Bayer AG, Leverkusen, Germany, batch no EQ13001804). Target application rates were 100 mg/m^2 for broflanilide (BRF), 1000 mg/m^2 for pirimiphos-methyl (PMM), 200 mg/m^2 for clothianidin (CTD), and 25 mg/m^2 for deltamethrin (DLT). A different spray tank (Micron CS14) was used for each insecticide product. For both application methods, the spray tanks were positioned stationary on the floor, which differs from the WHO guidelines for manual spraying where the tank is typically carried over one shoulder. For each insecticide, four panels were sprayed with the track sprayer and four panels were sprayed manually (Figure 1). Filter papers (9 cm diameter Whatman No. 1) were fixed inside Petri dish lids with sticky tack; the lids were pierced in the centre and attached to the panel using shoe tacks. Filter papers were positioned in a grid as shown in Figure 1, with three horizontal and five vertical positions per swath. Each panel with 15 filter papers constituted one replicate test, resulting in four replicates per insecticide product.

2.4. Determining Spray Deposit Using a Fluorescent Tracer

Filter papers sprayed with a fluorescent tracer were removed from Petri dishes using tweezers and placed, with minimal handling, into individual labelled ziplock bags. 100 mL of 10% NaOH v/v solution was added to each bag and subsequently stored in the dark for 60 min. Each bag was agitated thoroughly for approximately 1 min to mix the solution and ensure all fluorescein had been extracted from the filter papers. Then, an aliquot of the sample was added to a glass test tube. Fluorescence of each sample was measured using a Sequoia–Turner Model 450 Fluorometer and fluorescein filter set with excitation at 490 nm and emission at 515 nm. The fluorimeter was calibrated before each replicate against known concentrations of fluorescein applied to filter papers. Before analysing samples, a single concentration standard was used to check for any drift in the fluorescence measured over time.

Fluorescence heat maps were generated using Microsoft Excel as a proxy for dosage applied. A three-colour format was used, with the lowest recorded fluorescence value as the minimum (yellow), the second highest recorded fluorescence value as the maximum (red), and a mid-point at 50% of the difference between the high and low points (blue) when recording concentration.

2.5. Insecticide Sprayed Filter Papers

Sprayed filter papers were left to dry in the experimental huts for a minimum of 24 h, before they were wrapped individually in aluminium foil and stored at 5 ± 3 °C. The concentration of active ingredient on the filter papers was determined using high-performance liquid chromatography (HPLC). Samples were extracted from the filter papers at KCMUCo, and dried extracts were shipped to the Liverpool School of Tropical Medicine (LSTM) for HPLC analysis. The HPLC analysis was performed on a Dionex UltiMate 3000 comprising of an autosampler, quaternary pump, and variable wavelength detector. Chromeleon 7.2 SR4 software was used for peak analysis.

Prior to extraction, 12 circles were punched out of the filter paper using a 0.635 cm radius (½ inch diameter) hole punch, to have a consistent exact surface area of 15.201 cm^2 per disc to extract the sample from. A volume of 5 mL of a 100 µg/mL DCP in acetone solution was pipetted into a glass tube containing each filter paper sample and sonicated for 15 min using an Ultrawave U500H Ultrasonic Cleaning Bath (4.5 litre). Then, 1 mL of the sonicated sample was transferred to a new vial and left to evaporate until dry.

Samples were re-suspended using 1 mL of HPLC grade acetonitrile, and vortexed for at least 1 min at 2500–3000 rpm. Subsequently, samples were centrifuged (Eppendorf Centrifuge 5430) at 13,000 rpm for 20 min, and directly afterwards 100 µL of each sample

was pipetted into individual HPLC vials. A 250 mm × 4.6 mm HPLC column (Thermo Scientific Hypersil Gold C18) was used for all active ingredients, using an injection volume of 20 µL. HPLC methods were tailored for each active ingredient as detailed in Table 1.

Table 1. HPLC methodology per active ingredient in the samples.

	Active Ingredient			
	Pirimiphos-Methyl	**Broflanilide**	**Deltamethrin**	**Clothianidin**
Particle size	5 µM	5 µM	5 µM	5 µM
Wavelength	232 nm	254 nm	232 nm	232 nm
Run time	22 min	22 min	9 min	9 min
Mobile phase	70% Acetonitrile: 30% water	70% Acetonitrile: 30% water	93% Acetonitrile: 7% water with 0.1% phosphoric acid	93% Acetonitrile: 7% water with 0.1% phosphoric acid
Flow rate	1 mL/min	1 mL/min	1 mL/min	1 mL/min

2.6. Statistical Analysis

Graphical output was generated using R version 4.0.5 using the ggplot2 package and Microsoft Excel. Spray data for both track and manual spraying were not normally distributed, even after transformation with either $\log_{10}$ or square root methods. Therefore, untransformed data were used with non-parametric tests for statistical analysis. Significance between spray categories was evaluated using the Kruskal–Wallis method, incorporating Dunn's correction for multiple comparisons, with an alpha of 0.05. Unpaired, 2-tailed *t*-test was used to compare time taken to spray a downwards swath vs. an upwards swath.

A fluorimetry calibration was performed with each trial, and data were corrected accordingly prior to analysis. The fluorescein spray data were analysed with and without overlap points, due to the assumed greater inherent variability in the overlap spray zones. Comparisons between the insecticide concentration and the target dose, and subsequently the corrected target dose, were performed using one-sample Wilcoxon signed rank tests. Analysis was performed using Microsoft Excel, R version 4.0.5, and GraphPad Prism v7.03.

3. Results

3.1. Fluorescein Spray Results

In total, 547 and 488 filter papers sprayed with the automated track sprayer were included for analysis with and without overlap points, respectively, and 475 and 375 filter papers were included for the manual spray.

Heat maps plotting the distribution of fluorescein deposition on the wall surface showed a general uniformity of spray from the automated track sprayer, with the majority of recorded values falling around the mid-point colour range; some visual variation was apparent between the Left, Centre, and Right swath positions (Figure 2A). Similar uniformity of deposition was not evident in the manual wall spray, which showed high variability of spray over the entire wall surface, particularly when fluorescein deposition at the top and bottom wall positions were compared (Figure 2B).

Descriptive statistical analyses confirmed that wall spraying using the track sprayer had higher median and mean fluorescein deposition, and lower standard deviation and percentage coefficient of variations in the track spray compared to the manual spray (Table 2, SD of 36.15 and 33.87 for track spray with and without overlaps, respectively, compared to 88.83 and 69.51 for manual spray with and without overlap, respectively, and coefficient of variation of 19.96% and 19.30% for track spray with and without overlap, respectively, vs. 58.03% and 53.54% for manual spray with and without overlap, respectively).

	L1	C1	R1	O1	L2	C2	R2	O2	L3	C3	R3	O3	L4	C4	R4	O4	L5	C5	R5
A	182.7	147.2	203.0		179.7	141.2	238.5		166.5	134.1	226.3		207.0	149.3	212.1		193.9	151.3	196.9
	241.1	133.8	160.6	183.3	190.5	150.3	171.9	147.2	196.7	136.8	177.1	114.1	210.1	129.6	186.4	152.3	202.9	145.1	174.0
	224.7	148.7	177.9	237.5	245.7	141.7	202.5	222.4	208.3	156.9	207.2	175.6	236.4	142.9	195.5	153.4	222.4	149.9	168.6
	229.6	161.3	183.3	222.3	234.5	180.8	267.4	227.2	222.3	162.6	202.8	190.6	241.8	158.9	193.0	249.1	207.7	161.3	197.9
	219.4	148.5	181.7	180.5	236.5	149.6	187.4	205.7	219.4	143.9	158.8	175.9	217.1	163.4	189.7	236.5	224.0	149.6	173.6
	180.9	155.1	167.5	170.6	210.8	143.7	183.0	178.8	229.4	142.7	180.9	173.7	231.5	165.4	196.4	162.3	218.1	148.9	170.6
B	L1	C1	R1	O1	L2	C2	R2	O2	L3	C3	R3	O3	L4	C4	R4	O4	L5	C5	R5
	179.7	139.1	196.9		169.5	135.1	205.0		189.8	135.1	222.3		200.0	152.3	206.0		151.3	153.3	218.2
	226.7	139.9	180.2	206.0	182.3	132.7	184.3	154.4	202.9	126.5	164.7	141.0	220.5	138.9	180.2	184.3	202.9	141.0	168.8
	221.2	151.1	177.9	234.0	222.4	142.9	187.3	228.2	242.2	160.4	186.1	174.4	251.6	149.9	188.5	187.3	230.5	149.9	186.1
	216.2	152.8	185.7	224.8	241.8	150.4	204.0	235.7	239.4	160.1	196.7	184.5	238.2	157.7	194.3	252.8	219.9	144.3	163.8
	205.7	135.9	158.8	188.5	213.7	142.8	166.8	204.5	219.4	137.1	173.6	177.1	204.5	133.6	170.2	197.7	206.8	138.2	172.5
	174.7	138.5	163.3	190.2	199.5	129.2	169.5	171.6	227.4	132.3	172.6	173.7	216.0	140.6	193.3	177.8	196.4	142.7	179.9
C	L1	C1	R1	O1	L2	C2	R2	O2	L3	C3	R3	O3	L4	C4	R4	O4	L5	C5	R5
	147.2	144.2	206.0		187.8	146.2	210.1		155.4	142.2	215.2		198.9	137.1	190.8		134.1	144.2	172.6
	206.0	134.8	169.9	202.9	196.7	130.7	173.0	199.8	192.6	131.7	181.2	143.0	206.0	123.4	193.6	201.9	191.6	119.3	177.1
	222.4	144.1	148.7	243.4	216.5	134.7	172.1	249.2	206.0	141.7	170.9	203.7	231.7	137.1	187.3	223.5	210.7	92.6	148.7
	216.2	151.6	176.0	238.2	235.7	133.3	200.4	245.5	222.3	162.6	202.8	206.5	257.7	151.6	201.6	271.1	222.3	126.0	184.5
	211.4	139.3	154.2	187.4	212.5	140.5	172.5	225.1	195.4	133.6	156.5	206.8	191.9	130.2	156.5	226.2	198.8	126.8	167.9
	178.8	133.4	150.9	188.1	209.8	117.9	167.5	195.3	218.1	119.9	160.2	169.5	216.0	126.1	190.2	188.1	206.7	136.5	162.3
D	L1	C1	R1	O1	L2	C2	R2	O2	L3	C3	R3	O3	L4	C4	R4	O4	L5	C5	R5
	156.4	170.6	241.5		190.8	144.2	229.3		192.9	146.2	240.5		198.9	150.3	201.0		153.3	162.5	169.5
	212.2	142.0	182.3	214.3	199.8	124.5	176.1	221.5	189.5	137.9	167.8	162.7	238.0	122.4	181.2	223.6	191.6	120.3	168.8
	194.3	145.2	180.3	243.4	208.3	131.2	161.6	224.7	203.7	126.5	169.8	192.0	236.4	134.7	176.8	206.0	218.8	144.1	93.8
	206.5	155.2	182.1	256.5	230.9	144.3	197.9	240.6	233.3	149.1	182.1	186.9	251.6	158.9	204.0	282.1	205.2	141.8	191.8
	196.5	134.8	161.1	173.6	216.0	131.3	151.9	217.1	195.4	127.9	150.8	218.2	175.9	126.8	181.7	193.1	185.1	129.1	166.8
	171.6	130.3	142.7	198.4	190.2	121.0	165.4	176.8	209.8	114.8	167.5	166.4	228.4	123.0	180.9	201.5	188.1	136.5	176.8
E	L1	C1	R1	O1	L2	C2	R2	O2	L3	C3	R3	O3	L4	C4	R4	O4	L5	C5	R5
	147.2	145.2	208.1		177.7	147.2	215.2		150.3	142.2	204.0		180.7	151.3	191.8				
	213.2	117.2	167.8	227.7	200.8	127.6	190.5	234.9	181.2	135.8	167.8	148.2	217.4	122.4	188.5	266.9	174.0	125.5	164.7
	202.5	158.1	169.8	236.4	222.4	131.2	176.8	246.9	218.8	142.9	160.4	221.2	231.7	123.0	207.2	287.8	241.0	179.1	165.1
	194.3	155.2	166.2	255.2	234.5	136.9	185.7	230.9	234.5	149.1	171.1	200.4	247.9	149.1	194.3	302.8	200.4	145.5	162.6
	191.9	134.8	158.8	165.6	201.1	116.5	149.6	260.6	187.4	131.3	149.6	212.5	179.4	131.3	162.2	248.0	171.4	127.9	148.5
	160.2	129.2	144.7	233.6	186.1	124.1	152.0	195.3	179.9	121.0	159.2	169.5	199.5	111.7	163.3	211.9	198.4	134.4	171.6

(**A**)

	L1	C1	R1	O1	L2	C2	R2	O2	L3	C3	R3	O3	L4	C4	R4	O4	L5	C5	R5
A	339.2	252.7	416.3	248.5	81.8	71.4	129.7	241.2	139.1	132.8	164.1	272.5	91.2	89.1	130.8	93.2	273.5	167.2	129.7
	240.1	214.7	212.2	341.4	166.6	103.3	157.7	256.5	229.9	186.9	223.6	295.8	83.0	69.1	126.1	288.2	131.1	117.2	148.9
	195.7	175.9	234.0	323.4	178.7	117.8	131.9	489.3	317.7	293.6	370.2	317.7	150.4	133.3	343.2	451.0	239.7	188.7	215.6
	97.1	151.2	370.9	219.1	114.4	122.4	115.5	270.8	138.5	144.3	254.7	335.3	98.3	89.1	228.3	182.3	85.6	94.8	100.6
	296.7	181.1	173.0	295.6	113.4	125.1	157.8	156.6	198.7	147.3	130.9	663.4	184.6	123.9	170.6	30.5	211.5	144.9	128.6
B	L1	C1	R1	O1	L2	C2	R2	O2	L3	C3	R3	O3	L4	C4	R4	O4	L5	C5	R5
	228.7	174.5	196.4	261.0	74.5	48.4	77.6	187.0	131.8	113.0	158.9	337.1	157.9	147.4	246.4	257.9	131.8	125.5	195.4
	181.8	164.1	202.1	269.2	136.2	112.1	140.0	200.8	222.3	155.2	181.8	270.5	113.4	95.7	153.9	224.9	229.9	152.7	146.3
	183.0	153.2	140.4	300.7	344.6	175.9	197.2	312.0	200.0	167.4	195.7	354.6	147.5	105.0	287.9	246.8	102.2	127.7	151.8
	71.8	89.1	139.7	109.8	247.8	94.8	124.7	330.7	129.3	108.6	109.8	219.1	75.3	66.1	205.3	273.1	123.6	101.7	182.3
	170.6	159.0	164.8	428.7	184.6	176.5	290.9	176.5	130.9	76.1	67.9	182.3	211.5	139.1	181.1	468.4	226.7	219.7	243.0
C	L1	C1	R1	O1	L2	C2	R2	O2	L3	C3	R3	O3	L4	C4	R4	O4	L5	C5	R5
	117.2	75.5	89.1	152.6	217.3	161.0	266.2	199.5	53.6	50.5	70.3	176.6	159.9	148.5	210.0	153.7	54.7	49.5	64.1
	119.7	103.3	133.7	226.1	129.9	86.8	104.5	147.6	133.7	93.1	81.7	156.5	266.7	128.6	155.2	246.4	202.1	150.1	175.5
	123.4	85.1	92.2	209.9	246.8	129.1	173.1	307.8	137.6	96.5	124.8	322.0	147.5	126.3	273.7	300.7	173.1	188.7	212.8
	59.1	82.2	92.5	374.4	212.2	186.9	257.0	244.4	91.4	91.4	97.1	274.3	83.3	102.9	224.8	242.1	90.2	92.5	140.8
	84.2	78.4	94.7	303.7	159.0	163.6	184.6	232.5	149.6	108.8	106.4	269.9	213.8	149.6	196.3	366.8	184.6	135.6	160.1
D	L1	C1	R1	O1	L2	C2	R2	O2	L3	C3	R3	O3	L4	C4	R4	O4	L5	C5	R5
	100.5	62.0	56.8	111.0	249.6	170.4	199.5	120.3	74.5	69.3	66.1	208.9	169.3	163.1	223.5	159.9	53.6	32.8	44.3
	70.3	55.1	64.0	189.4	160.3	93.1	86.8	197.0	166.6	60.2	62.7	191.9	218.5	126.1	155.2	304.7	146.3	100.7	95.7
	75.2	63.9	55.4	225.5	148.9	89.4	112.1	286.5	103.6	83.7	117.8	398.5	197.2	167.4	356.0	225.5	92.2	90.8	75.2
	79.9	79.9	69.5	216.8	106.3	68.4	113.2	286.9	70.7	67.2	81.0	240.9	73.0	61.5	143.1	193.8	54.5	45.3	41.9
	115.8	101.7	108.8	387.8	157.8	121.6	160.1	196.3	93.6	71.4	62.0	268.7	251.2	191.7	244.2	383.1	104.1	69.1	55.0
E	L1	C1	R1	O1	L2	C2	R2	O2	L3	C3	R3	O3	L4	C4	R4	O4	L5	C5	R5
	31.8	29.7	28.6	68.2	223.5	79.7	155.8	104.7	52.6	38.0	39.1	152.6	150.6	84.9	84.9	171.4	57.8	33.8	28.6
	8.2	10.8	56.4	85.5	90.6	62.7	74.1	162.8	133.7	36.1	34.8	102.0	56.4	102.0	112.1	145.1	71.6	43.7	43.7
	14.2	17.1	52.5	112.1	99.3	73.8	133.3	139.0	110.7	44.0	76.6	209.9	122.0	112.1	146.1	123.4	29.8	21.3	46.8
	32.7	29.2	93.7	132.8	128.2	89.1	117.8	224.8	52.2	39.6	102.9	249.0	127.0	54.5	114.4	177.7	46.5	39.6	36.1
	60.9	60.9	66.7	225.5	143.8	83.1	101.7	196.3	36.4	30.5	58.5	155.5	114.6	51.5	90.1	198.7	100.6	53.9	42.2

(**B**)

Figure 2. Heat maps of fluorescein deposition on wall surfaces using an automated track (**A**) and manual (**B**) spray. Filter papers to collect fluorescein deposited by each spray type were attached to walls in the configuration shown. Five swaths were present on each wall, measured at the Left (L), Centre (C), Right (R), and Overlap (O) positions. Letters A–E indicate the height position down the wall. Six trials were performed for the track spray and five were performed for the manual spray. Individual cells show the corrected fluorescence values for each spray trial replicate. Orange indicates higher fluorescein values, blue indicates midpoint values, and yellow indicates low values. A far higher degree of variation in fluorescein deposition is apparent in the manual spray compared to the track spray.

Including the overlap positions resulted in a significant different dataset for manual spraying ($p \leq 0.0001$), but not for the track sprayer dataset ($p = 0.6998$). Both sets of manual spray data were significantly different to the track spray datasets (4 comparisons, $p \leq 0.0001$ in all cases). Analysis of each combination of vertical and horizontal swath positions showed greater variation of fluorescein deposition in the manual spray at every wall position point compared to the track sprayer.

Table 2. Descriptive statistics for manual vs. track spraying using fluorescein. Results are given both with and without swath overlap positions.

Descriptive Statistic	Manual Spray Including Overlap	Manual Spray Not Including Overlap	Track Spray Including Overlap	Track Spray Not Including Overlap
Minimum	8.247	8.247	92.65	92.65
Maximum	663.4	416.3	302.8	267.4
Median	137.6	121.6	180.2	172.8
Mean	153.1	129.8	181.1	175.5
SD	88.83	69.51	36.15	33.87
%CV	58.03%	53.54%	19.96%	19.30%

3.2. Insecticide Spray Results

For both pirimiphos-methyl (PMM) and broflanilide (BRF), 60 filter papers were sprayed with the track sprayer and 60 with manual spraying. As a dual AI formulation, the concentration of both deltamethrin (DLT) and clothianidin (CTD) was determined in the same filter papers; 57 for manual spraying and 60 for the track sprayer. The concentration of active ingredient on each filter paper was visualised in boxplots (Figure 3). Similar to spraying with fluorescein in laboratory conditions, variation in insecticide application rate in the experimental huts was much greater for manual spraying compared to the track sprayer. For both PMM and BRF, Levene's test reported unequal variances between manual spraying and the track sprayer ($F = 3.316$, $p < 0.001$ and $F = 4.3533$, $p < 0.001$, respectively), indicating that the variation in insecticide application rate from top to bottom and from left to right of the wall was larger when spraying manually compared to using the automated track sprayer. Likewise, for CTD and DLT, the vertical variance between the two application methods was statistically different ($F = 4.1735$, $p < 0.001$ and $F = 4.6389$, $p < 0.001$ respectively).

Two of the active ingredients, PMM (Figure 3A) and BRF (Figure 3B), showed a significant difference ($p < 0.01$ and $p < 0.0001$, respectively) in the sprayed concentration when comparing track and manual spraying. For BRF, this resulted in a lower median concentration for the track sprayer (66.0 mg/m^2, SD 23.8) compared to manual spraying (91.4 mg/m^2, SD 49.7), whereas for PMM the median track sprayer concentration was higher (485.8 mg/m^2, SD 193.4) compared to manual spraying (392.3 mg/m^2, SD 268.6). Clothianidin (CTD) and deltamethrin (DLT) were sprayed together in the combination product Fludora Fusion and, unsurprisingly, the results for the two actives followed the same pattern and trend (Figure 3C,D). The amount of CTD sprayed by manual application (177.9 mg/m^2, SD 51.3) was not significantly different from the amount of CTD sprayed using the track sprayer (165.4 mg/m^2, SD 104.7), $p = 0.5288$. Likewise, the amount of DLT sprayed by manual application (18.4, SD 11.0) was not significantly different from the amount of DLT sprayed by using the track sprayer (19.1, SD 5.1), $p = 0.6217$.

For each active ingredient, the amount delivered by each spray method was also compared to the target dose. For PMM the target dose is 1000 mg/m^2, and both spray methods resulted in a significantly lower dose on filter papers ($p < 0.0001$). The concentration found in the liquid samples taken from the spray tanks prior to spraying was used to correct for possible mixing errors (see Table 3). This resulted in calculated concentrations of 568.5 mg/m^2 for manual spraying and 576.6 mg/m^2 for the track sprayer. Compared to the corrected target dose, manual spraying was significantly lower ($p < 0.001$), but there was no significant difference for the track sprayer ($p = 0.07913$).

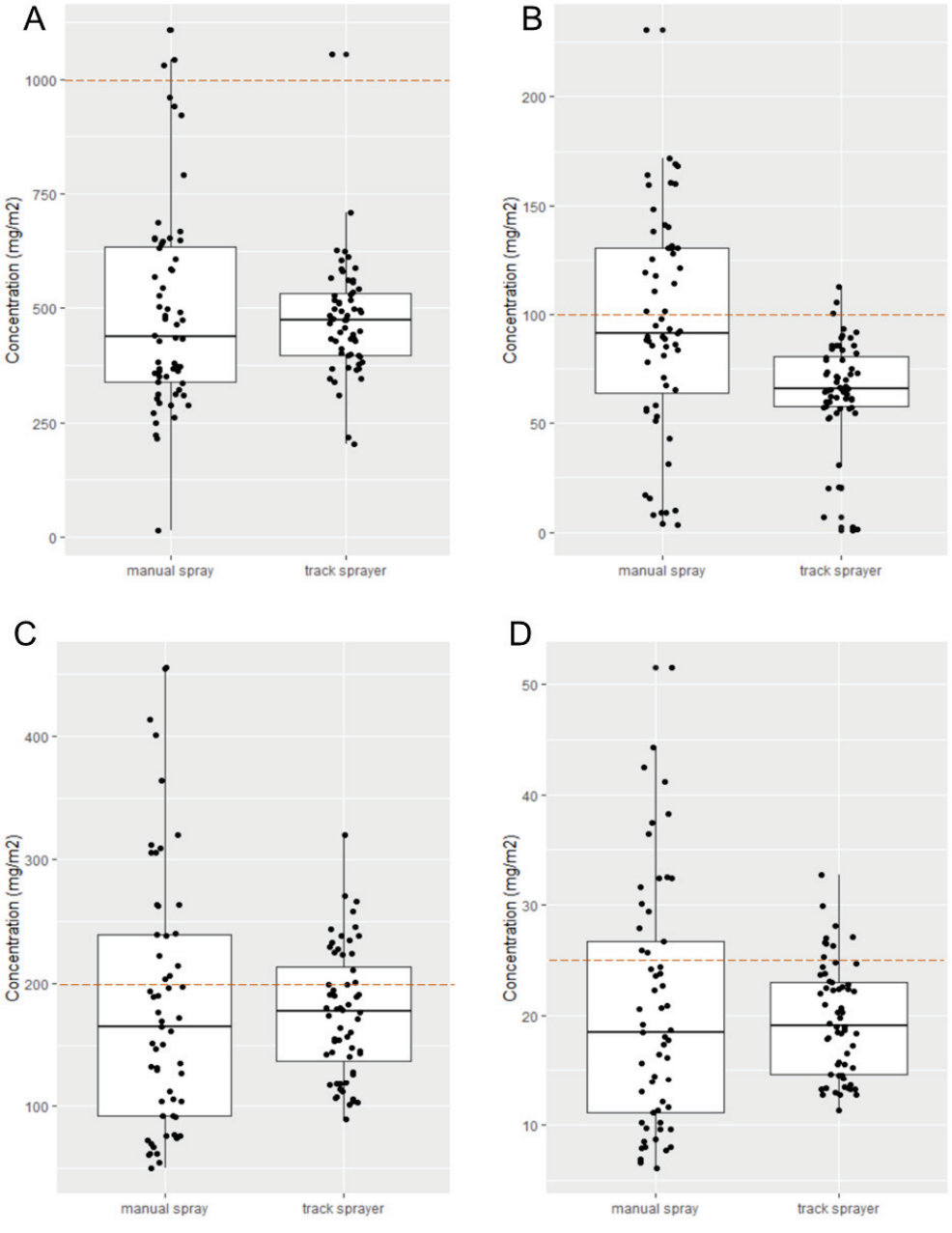

Figure 3. Concentration of active ingredient on filter papers sprayed by manual spraying or the automated track sprayer. Filter papers were attached to plywood panels in a grid of 15 per swath. Four swaths were treated for each spray method per insecticide, resulting in 120 filter papers per insecticide. Letters A–D indicate the active ingredients; pirimiphos-methyl (**A**), broflanilide (**B**), clothianidin (**C**), and deltamethrin (**D**). Individual dots show the values for each filter paper. Boxplots indicate median, 25th and 75th percentile, extreme lines, and potential outliers. Dashed horizontal lines represent the target concentration for each insecticide.

For BRF, the amount of active ingredient sprayed on filter papers was not significantly different from the target dose for manual spraying ($p = 0.4034$, and $p = 0.2403$ for the corrected dose), but was significantly lower for the track sprayer ($p < 0.0001$). Similarly, for CTD, the dose applied to filter papers by manual spraying was not significantly different from the target dose ($p = 0.0676$, and $p = 0.8863$ for the corrected dose), but the dose applied by the track sprayer was significantly lower ($p < 0.001$, and $p < 0.01$ for the corrected dose). For DLT manual spraying was significantly different from the target dose of 25 mg/m^2 ($p < 0.01$) but not after correcting for the concentration in the spray tank ($p = 0.9051$). The track sprayer resulted in a significantly lower dose, both corrected and uncorrected ($p < 0.0001$).

3.3. HPLC Analysis of Liquid Samples

Samples of insecticide solutions were taken directly from the spray tank before and after spray application for both application methods to be able to detect non-homogeneous mixing. Deviation from the target concentration was calculated for each active ingredient (Table 3). The target concentrations for the liquid samples were calculated by taking the recommended dose per m^2, divided by the application rate of 30 mL/m^2, resulting in a target concentration of 33.33 mg/mL for PMM, 6.66 mg/mL for CLT, 0.83 mg/mL for DLT, and 3.33 mg/mL for BRF. Apart from the concentration of BRF before spraying, the concentration of active ingredient found in the spray solution was generally lower than the target concentration. The spray tank solution had a DLT concentration of 0.65 mg/mL before and 0.71 mg/mL after manual spraying (target 0.83), and a CTD concentration of 5.66 mg/mL before and 6.15 mg/mL after spraying (target 6.66 mg/mL). The average concentration of BRF was 3.44 mg/mL before and 3.06 mg/mL after spraying, both within 10% of the target concentration (3.33 mg/mL). The concentration of PMM in the tank solution was considerably lower than the target dose (33.3), ranging between 19.22 mg/mL (−36%) and 15.11 mg/mL (−50%).

Table 3. HPLC results of liquid samples taken from the spray tank before and after spraying. The target dose in mg/m^2 is given for each active ingredient. HPLC results are represented as a percentage deviation from the target dose.

		Concentration before	Concentration after	Target Concentration	Deviation from Target Dose
PMM	Manual	18.95 mg/mL	15.11 mg/mL	33.33 mg/mL	−37% to −50%
	Track	19.22 mg/mL	16.18 mg/mL	33.33 mg/mL	−36% to −46%
CLT	Manual	5.66 mg/mL	6.15 mg/mL	6.66 mg/mL	−8% to −15%
	Track	6.59 mg/mL	6.60 mg/mL	6.66 mg/mL	1%
DLT	Manual	0.65 mg/mL	0.71 mg/mL	0.83 mg/mL	−14 to −22%
	Track	0.79 mg/mL	0.77 mg/mL	0.83 mg/mL	−5% to −7%
BRF	Combined	3.44 mg/mL	3.06 mg/mL	3.33 mg/mL	3% to 8%

3.4. Consistency of Wall Spraying across the Swath

The nozzle delivered the most consistent fluorescein deposition from the track sprayer at the centre of the swath, displaying the lowest range of fluorescein deposited, the lowest standard deviation (13.43 vs. 24.25, 22.36 and 35.36 for Centre, Left, Right, and Overlap positions, respectively), and the lowest coefficient of variation (9.58% vs. 11.79%, 12.37% and 17.15% for Centre, Left, Right, and Overlap positions, respectively). However, significantly less fluorescein was deposited in this position compared to both the left and the right positions on the swath ($p \leq 0.0001$ in both cases, Figure 4A). Further significant differences were seen between the Left and Right, Right and Overlap, and Centre and Overlap positions ($p \leq 0.0001$ for all). No significant difference was seen between the Left and the Overlap positions ($p \geq 0.9999$).

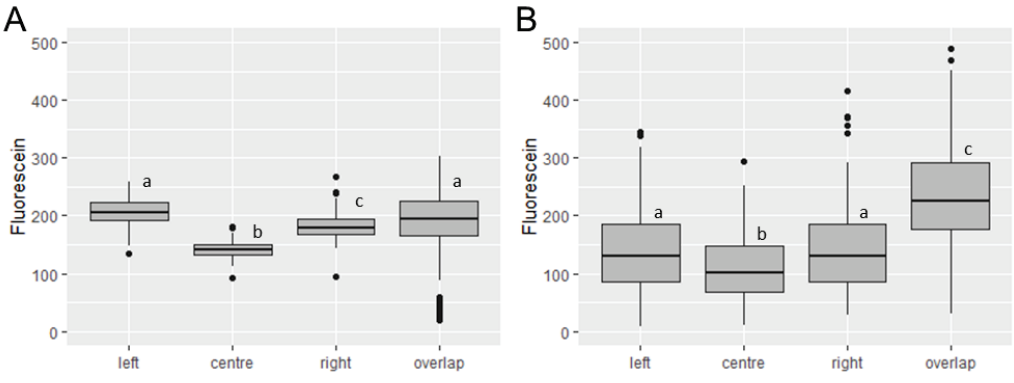

Figure 4. Box and whisker plots of horizontally stratified fluorescence. Track (**A**) and Manual (**B**) spray data was classified using left, centre, right, and overlap horizontal swath positions. The centre position on the track sprayer showed the most consistent fluorescein deposition. Greater differences were seen between horizontal positions on the track sprayer than the manual spray. Significant differences are indicated by different lowercase letters.

In contrast, there was no significant difference seen between the left and right swath positions when spraying manually ($p \geq 0.9999$, Figure 4B). However, significant differences were seen between Left vs. Centre ($p = 0.0041$), Right vs. Centre ($p = 0.0027$), Left vs. Overlap ($p = 0.0001$), and Right vs. Overlap ($p \leq 0.0001$) positions on the swath.

To discern whether the greater variation between fluorescein deposition at different wall heights in the manual spray was obscuring horizontal differences, data were further split into vertical and horizontal spray position categories. Except for comparisons including Overlap data, only one comparison showed a significant difference (CB vs. RB, $p = 0.048$). Plotting the stratified data showed a general trend for mean values from the Centre position to be lower than either Left or Right position data, indicating that this variation exists independently of the method of spray application.

Similar to results with fluorescein, the amount of insecticide sprayed with the track sprayer was most consistent in the middle position of a swath (Table 4). For both CLT and DLT, the middle position also had the lowest concentration deposited ($p < 0.0001$ for all combinations). However, this was not observed for PPM or BRF. For three out of the four insecticides, the left position on a swath resulted in a higher concentration of active ingredient applied compared to either the centre or right positions.

Table 4. Horizontally stratified application rates of insecticides. Manual and track spray data was classified using left, centre, and right horizontal swath positions. Median application rate ± standard deviation is indicated for each horizontal position. Comparisons are done between horizontal positions by spray method. Significant differences are indicated by different lower-case letters.

		Left	Centre	Right
BRF	Manual	47.0 ^{a} ± 40.0	93.1 ^{b} ± 34.4	122.4 ^{c} ± 39.0
	Track	66.2 ^{a} ± 25.1	61.2 ^{a} ± 15.9	85.0 ^{b} ± 25.8
PMM	Manual	300.3 ^{a} ± 266.7	400.9 ^{ab} ± 233.9	446.0 ^{b} ± 292.1
	Track	782.6 ^{a} ± 228.5	462.5 ^{b} ± 67.2	467.9 ^{b} ± 138.5
CTD	Manual	205.7 ^{a} ± 134.4	151.7 ^{b} ± 80.4	168.8 ^{ab} ± 85.5
	Track	224.7 ^{a} ± 45.4	118.8 ^{b} ± 56.1	181.6 ^{c} ± 32.1
DLT	Manual	23.1 ^{a} ± 13.8	17.4 ^{a} ± 8.4	18.3 ^{a} ± 9.7
	Track	23.3 ^{a} ± 4.2	14.0 ^{b} ± 5.2	20.8 ^{c} ± 3.8

Greater variation in spraying was observed when spraying manually for all horizontal positions for all active ingredients, except for PMM in the left position. For PMM and BRF, there was a trend from left to right with a lower amount deposited at the left compared to the right position on a swath ($p < 0.05$ for PMM and $p < 0.0001$ for BRF). No significant differences were found for DLT. For CLT, the left position showed a significantly higher concentration of active ingredient applied compared to the middle position on a swath ($p < 0.05$).

3.5. Consistency of Wall Spraying along the Swath

Although the difference was slight, there was a general trend for fluorescein deposition from the track sprayer to be greater at the top of walls, decreasing with each vertical wall position (Figure 5). There were no significant differences between deposition onto filter papers immediately above or below each other. However, a significant difference between deposition onto filter papers at the uppermost and lowermost wall positions was evident (high vs. low, $p = 0.0166$).

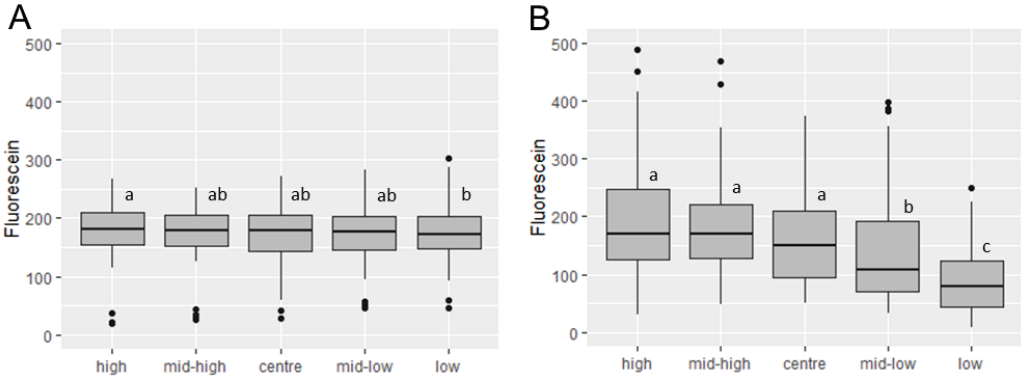

Figure 5. Wall height analysis for track and manual spray with fluorescein. (**A**) Track spray stratified by height with no overlap; (**B**) Manual spray stratified by height with no overlap. Significant differences are indicated by different lowercase letters.

Fluorescein deposition using a manual spraying (Figure 3B) showed significant differences between the two upper positions and the two lowest positions on the wall (four comparisons, $p \leq 0.0001$). Significant differences in fluorescence were seen between the centre and the lower middle position ($p = 0.0294$), centre and bottom position ($p \leq 0.0001$), and lower middle and bottom position on the wall ($p = 0.0017$). No significant differences were seen between the three upper positions. When the direction of spray (upwards or downwards swath) and total swath spray time were added to the analysis, the pattern of variation was preserved only in downwards swaths. Upwards swaths showed no significant difference between fluorescein deposition at the tops and bottoms of walls (p= 0.9555), but significant differences were seen between the top and centre of the wall ($p = 0.0164$) and the upper middle, middle, and lower middle compared to the bottom of the wall ($p = 0.0014$; $p \leq 0.0001$; $p = 0.0356$, respectively). No significant difference was found between the time taken to spray a downwards swath vs. an upwards swath ($p = 0.8779$), indicating that it is spray rhythm, rather than spray time, that differs with spray direction.

The minor trend towards decreasing fluorescence with vertical positions was not shown when spraying with insecticides. No significant differences were found between vertical positions sprayed with the track sprayer for either PMM or BRF. For DLT and CLT, only the upper middle position was significantly different, compared to the lower middle position (CLT; $p < 0.05$) or the centre position (DLT; $p < 0.05$).

Variation in insecticide application rate when sprayed manually was generally larger by vertical position compared to the track sprayer. Although some significant differences were found, there was no common trend between insecticides (Table 5). For PMM, the centre position had a significantly lower concentration on filter papers compared to the upper middle and lowest position (two comparisons; $p < 0.05$). No significant differences were found for BRF. For DLT, only the highest position was significantly different, compared to upper middle, centre, and lower middle positions (three comparisons, $p < 0.01$) and lowest position ($p < 0.001$). Similarly, for CLT the highest position was significantly different, compared to upper middle and centre positions (two comparisons; $p < 0.01$) lower middle position ($p < 0.05$), and lowest position ($p < 0.001$).

Table 5. Wall height analysis for track and manual spray for insecticides. Median application rate ± standard deviation is indicated for each vertical position. Comparisons are done between vertical positions by spray method. Significant differences are indicated by different lowercase letters.

		High	Mid-High	Centre	Mid-Low	Low
BRF	Manual	98.2 ^{a} ± 58.8	90.7 ^{a} ± 46.9	105.5 ^{a} ± 53.2	95.2 ^{a} ± 44.7	76.0 ^{a} ± 54.4
	Track	67.5 ^{a} ± 20.5	68.5 ^{a} ± 14.9	65.3 ^{a} ± 28.6	72.0 ^{a} ± 35.7	61.8 ^{a} ± 17.7
PMM	Manual	334.0 ^{ab} ± 169.4	538.3 ^{b} ± 317.8	215.1 ^{a} ± 172.5	421.1 ^{ab} ± 268.1	421.8 ^{b} ± 330.0
	Track	510.0 ^{a} ± 223.4	500.0 ^{a} ± 194.3	474.7 ^{a} ± 219.3	498.0 ^{a} ± 175.0	463.8 ^{a} ± 166.6
CTD	Manual	263.2 ^{a} ± 77.3	152.4 ^{b} ± 91.3	161.8 ^{b} ± 125.7	140.8 ^{b} ± 104.5	104.0 ^{b} ± 71.5
	Track	159.7 ^{ab} ± 53.9	148.8 ^{a} ± 36.6	194.9 ^{ab} ± 53.8	185.4 ^{b} ± 58.1	180.9 ^{ab} ± 46.5
DLT	Manual	31.6 ^{a} ± 8.8	16.8 ^{b} ± 9.9	19.2 ^{b} ± 12.8	16.4 ^{b} ± 9.5	11.7 ^{b} ± 7.4
	Track	17.0 ^{ab} ± 4.9	17.2 ^{a} ± 3.7	22.4 ^{b} ± 5.2	20.5 ^{ab} ± 5.9	19.6 ^{ab} ± 5.1

4. Discussion

IRS is a widely applied vector control intervention. However, relatively little attention is given to the assessment of application rates whether at the level of control programmes and communities, or at the level of households. Tools aimed at improving consistency of application on a community level, such as the IK Smart Light [17], are being developed but are not yet widely deployed. Apart from the implications for IRS campaign success, consistent delivery of insecticidal products is important when conducting experimental hut trials, which are reliant on the well-defined application rate of IRS products. In this study, we compared an automated track spray system for IRS to a well-trained human spray operator, to discern whether the track sprayer delivered a more consistent spray such that use in experimental hut trials could be implemented.

Large variation in spray deposits, measured by the amount of insecticide applied to a filter paper, have previously been reported for the three insecticides tested in this study, ranging from 0.31 to 3.78 times the recommended dose for Actellic 300CS [18], between 0.63 and 1.37 times the dose for Vectron T500 [12,13], and between 0.80 and 1.32 for Fludora Fusion [19,20]. A large proportion of this variation could be removed by using an automated spraying process, such as shown here with the Micron track sprayer. Analysis of the overall wall spray pattern using both fluorescein and IRS products demonstrated markedly less variation in the automated than in the manual spray. This confirms that a significant proportion of variation in spray application could be eliminated. Improved accuracy in insecticide application in trials would lead to more robust data and thus better-informed product development decisions, which could avoid unnecessary delays in bringing new products to the market.

With the exception of the study with BRF, the track sprayer resulted in a higher median concentration sprayed than manual spraying. For the insecticidal products, results were also compared to the target dose and showed underdosing with both spray methods. Although this underdosing was only significant for the track sprayer, it is likely that the

underdosing was a factor for both methods, whilst the larger variation in manual spraying masked the difference between actual and target dose. Underdosing can occur if the speed of spraying is too fast, i.e., not enough liquid is deposited on the filter papers, or if the distance from the wall is too large. As both the distance and the speed are regulated for the track sprayer, it is unlikely that these factors caused the lower-than-expected application rate on the filter papers. It is recommended that in future studies, the track sprayer is used in conjunction with enhanced filter paper analysis so that any differences in the dose delivered compared to the target dose can be identified and evaluated.

To correct for potential deviations from target dose in the spray solution prior to spraying, we compared the sprayed filter papers to the concentration in liquid samples taken from the spray solution. Theoretically, the amount of insecticide sprayed onto filter papers would reflect the concentration of insecticide in the spray tank, assuming the spray nozzle moves at a constant speed up or down a swath and that the distance of the nozzle from the wall is also constant. Analysis of the spray solution can indicate dilution or mixing errors, such as adding too much or too little water or product to the spray tank or not shaking the spray tank to thoroughly mix the product with the water before and during spraying. We found that the concentration of PMM in the spray tank was considerably lower than expected, which may explain the lower-than-expected concentration found on filter papers with both spray methods.

Analysis of fluorescein values stratified by horizontal swath position showed that the 8002 nozzle used did not provide a consistent application rate across the horizontal swath, with less fluorescein being deposited in the centre of a swath. This difference was significant in the track sprayer deposits, but less apparent in the manual spray deposits. Similar to the results with fluorescein, the insecticide dose sprayed with the track sprayer was lower but more consistent in the centre position compared to positions at the edges of the swaths. Whilst the same nozzle was used for the track and manual spraying, different nozzles were used for fluorescein spray and each of the insecticidal products, making the possibility that this result could be an artefact of individual nozzles less likely.

We discerned a difference in spray rhythms in upward and downward swaths with manual spraying, even when the overall swath spray times were consistent between the two directions. The study was conducted using only one spray operator for each experiment and did not have the aim of characterising the entire range of variation that might be present during manual spraying. However, it is interesting to note that, even with expert training, and when keeping to the overall requirement of spraying 2 metres per 5 s, differences in rhythm can exist that lead directly to inconsistent spray application.

Height position analysis demonstrated significant differences in the amount of fluorescein applied between different wall height positions in the manual spray, particularly in the lower half of the wall. This aligns with the observation that spray operators tend to move the spray lance slowly at the top of the swath and then speed up towards the bottom. Although one significant difference was detected in fluorescein applied by the track spray between the uppermost and lowermost wall heights, no significant differences were seen when the overlap positions were also included, demonstrating that the track sprayer delivers a much more consistent spray application than the manual spray. Variation in the amount of insecticide sprayed manually was generally larger per vertical position compared to the track sprayer, but the trend of decreasing concentrations with lower wall heights was not shown when spraying with insecticides.

Overall, the track sprayer delivered a more consistent deposit of spray solution, making it a potential methodological improvement to experimental hut evaluations of novel IRS formulations.

5. Conclusions

Large variation was found in the amount of fluorescein and insecticide applied when following WHO guidelines for manual spraying by well-trained spray operators. When comparing the automated track sprayer to standard manual spraying, variation in applica-

tion rates were significantly reduced in all instances, indicating that a large proportion of the variation in spray application can be eliminated by automating the spraying procedure.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The dataset generated and analysed during this study is available from the corresponding author upon reasonable request.

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The Automatic Classification of Pyriproxyfen-Affected Mosquito Ovaries

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Simple Summary: With resistance to the pyrethroid family of insecticides increasing, it is more important than ever that tools are available to measure the efficacy of alternatives. Pyriproxyfen (PPF) is an alternative insecticide whose mode of action sterilises adult mosquitoes. Consequently, the efficacy of PPF-based tools can be measured through visual examination of egg development by trained experts. This, however, can be a time-consuming process for which the required expertise can be difficult to train and is not available in many contexts. As such, we propose that an objective machine learning program, which can automatically classify the fertility status of adult mosquito ovaries via a colour image, be developed to improve the speed, accuracy, and consistency of assessment. This study shows that a convolutional neural network, built in Python's TensorFlow library, can quickly classify images of dissected ovaries into either 'fertile' or 'infertile' with a high accuracy rate. Such an application would be a practical and accessible tool available to all researchers studying the efficacy of PPF or other insecticides with a similar mode of action.

Abstract: Pyriproxyfen (PPF) may become an alternative insecticide for areas where pyrethroid-resistant vectors are prevalent. The efficacy of PPF can be assessed through the dissection and assessment of vector ovaries. However, this reliance on expertise is subject to limitations. We show here that these limitations can be overcome using a convolutional neural network (CNN) to automate the classification of egg development and thus fertility status. Using TensorFlow, a resnet-50 CNN was pretrained with the ImageNet dataset. This CNN architecture was then retrained using a novel dataset of 524 dissected ovary images from *An. gambiae* s.l. *An. gambiae* Akron, and *An. funestus* s.l., whose fertility status and PPF exposure were known. Data augmentation increased the training set to 6973 images. A test set of 157 images was used to measure accuracy. This CNN model achieved an accuracy score of 94%, and application took a mean time of 38.5 s. Such a CNN can achieve an acceptable level of precision in a quick, robust format and can be distributed in a practical, accessible, and free manner. Furthermore, this approach is useful for measuring the efficacy and durability of PPF treated bednets, and it is applicable to any PPF-treated tool or similarly acting insecticide.

Keywords: *Anopheles* mosquito; fertility; ovary development; pyriproxyfen (PPF); side-effects; machine learning; image classification; automated identification; convolutional neural network

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1. Introduction

Insecticide-treated nets (ITNs) are a common vector control tool and have considerably decreased the burden inflicted by malaria [1]. However, in recent years, species of the

mosquito genus *Anopheles*, the principal vector for malaria, have demonstrated an increased resistance to the pyrethroid-based insecticides used to treat ITNs. This increase in resistance to pyrethroids threatens the efficacy of ITNs and may have contributed to an increase in malaria cases in affected areas [2]. Consequently, alternative effective insecticides for use on ITNs need to be identified to maintain the efficacy of this intervention and meet the gap in global disease control that pyrethroid resistance has created [3,4]. ITNs treated with a mixture of pyriproxyfen (PPF) and pyrethroids offer an alternative to standard pyrethroid-treated ITNs in areas where pyrethroid-resistant malaria vectors are prevalent [5–8]. The mode of action of PPF affects the fertility, longevity, and lifetime fecundity of malaria vectors [9,10], and PPF-treated ITNs have been shown to sterilise *Anopheles* mosquitos under both laboratory and field conditions [11,12]. As vector ovary development is inhibited by exposure to PPF [8], and females that fail to develop morphologically normal eggs have been shown to not oviposit [13,14], a means of measuring efficacy and monitoring the durability of PPF and PPF-treated tools is through the assessment of eggs for signs of abnormal or inhibited development [8,12]. Although different means of scoring sterility exist (e.g., by looking for the ability to prevent egg laying or oviposition inhibition), another method to determine fertility status is based on trained experts manually dissecting ovaries and classifying egg development according to Christopher's stages [15]. However, this can be a time-consuming process and requires a level of expertise not always available. Therefore, to increase the throughput and robustness of data used to measure the efficacy and durability of PPF-based ITNs, and to aid efficient and reproducible data collection in research settings, freely available alternative methods for the accurate, quick, and automatic classification of ovary development are required.

In recent years, deep learning models and convolutional neural networks (CNNs) have made significant progress across a range of computer vision problems, including image classification [16]. A CNN implements a convolution operation across several distinct layers to convert an input (i.e., an image) into an output (i.e., a classification). The convolution operation applies a filter or kernel (usually a 3×3 or 5×5 matrix) to a two-dimensional representation of an image. This matrix then slides over the full 2D grid, performing calculations on the data depending on the kernel's weights, transforming data into a representation of patterns found within the image (i.e., edges, etc.) [17]. A CNN, therefore, uses linear regression with forward and backward propagation in a neural network to automatically adjust and determine the most appropriate kernel weights [18,19]. These weights can then identify different pattern types found within a dataset, with layers earlier in the network identifying primitive features in an image, such as edges and colours, while deeper layers detect more complex shapes, patterns, or objects [20,21].

This type of architecture enables the automatic training and detection of multiple visual features, which can then be used to identify and classify variance between images. However, the area of application for deep learning and CNNs has been constrained by its reliance on large datasets to avoid overfitting (i.e., to ensure generalisability) and, thus, achieve high accuracy rates [22]. Nevertheless, the size of a dataset can be increased through data augmentation, which employs a raft of tactics so as to artificially increase the available dataspace and allow generalisable models to be built. Data augmentation includes the geometric transformation, colour augmentation, and random cropping of available data (amongst other techniques), thereby creating randomised novel images from those that are already available [23]. However, even with data augmentation, most datasets are still insufficient to avoid overfitting. In such cases, transfer learning can be used, whereby opensource architectures and pretrained weights, derived using very large datasets, are repurposed and fine-tuned for a different but related task [24]. Models trained against the ImageNet dataset (which contains over 14 million images and 20 thousand classes) are freely available and regularly achieve high levels of accuracy [25]. Three common and high-performing models used in transfer learning, all pretrained and tested against the ImageNet dataset, are (1) VGG-16 [26], (2) ResNet-50 [27], and (3) InceptionV3 [28,29].

Machine learning has already been successfully utilised within entomology for a number of species classification tasks, such as the identification of pest insect species [30], the recognition of lepidopteran species [31], and the classification of mosquito species [32–35]. Additionally, automatic tools have been developed to count the eggs laid by female mosquitos, which can be used to estimate fecundity [36–38]. However, current work on the automatic classification of mosquito fertility and egg development is limited. As such, this study is aimed at bridging this gap and uses deep learning, data augmentation, and transfer learning to develop a quick, robust, and practical method to classify the fertility status (i.e., 'fertile' or 'infertile') of mosquito ovaries from colour images. To be successful, this new method must (1) be automatic and require no, or limited, expert knowledge to categorise an image, (2) achieve close to the human accuracy rate of 99–100% (rate determined by the agreement between two scorers assessing the dataset used in this study), (3) be in an easily distributable, non-proprietary, and low-cost format, and (4) classify ovary fertility of an image faster than the estimated 2 s taken by human experts (rate determined by the mean time taken for four trained technicians to classify 30 random ovary images).

Using a novel dataset of dissected ovary images, data augmentation, and transfer learning, we were able to build and train a CNN in TensorFlow that can detect and classify the development status ('fertile' or 'infertile') of 157 ovaries in 38.5 s at a 94% accuracy rate. As such, this study proposes a new method for the automatic classification of the fertility status of *Anopheles* mosquito ovaries that is quick, accurate, and easily distributable, and that is not dependent on trained experts to score egg development.

2. Materials and Methods

2.1. Image Dataset

As no publicly available datasets exist, data from ongoing research were used for this study. A total of 524 images of dissected ovaries from 5–8 day old female mosquitos were collected and labelled with the appropriate fertility status (based on Christopher's stage of egg development). These were all full colour images obtained from three sources where fertility status was determined and corroborated by two trained expert scorers. A summary of the datasets used here is found in Table 1.

Table 1. Image dataset summary.

Dataset	Source	Strain	Image Count	Fertile	Infertile
1	Cove, Benin	*An. gambiae* s.l.	124	79	45
2	Insectary colony	*An. gambiae* Akron	187	43	144
3	Mwanza, Tanzania	*An. funestus* s.l.	125	67	58
4	Mwanza, Tanzania	*An. gambiae* s.l.	88	38	50
	Total		524	227	297

The first dataset contained a total of 124 blood-fed adult pyrethroid-resistant female *An. gambiae* s.l. mosquitoes which had survived exposure to either a control untreated net or a PPF-treated net (Royal Guard) in experimental hut studies performed in accordance with current WHO guidelines [39]. Mosquitoes were collected as wild free-flying adults in experimental huts in Cove, Southern Benin, with 36.3% (n = 45) classified as being infertile and the remaining 63.7% (n = 79) classified as being fertile. The second dataset contained 187 blood-fed adult pyrethroid-resistant female *An. gambiae* Akron mosquitoes from insectary-maintained colonies. All samples had survived exposure to either a control untreated net or a PPF-treated net (Royal Guard) in WHO cone bioassays [39]. Of the total samples in the second dataset, 77.0% (n = 144) were classified as being infertile, and 23.0% (n = 43) were classified as being fertile. All samples in the first and second dataset were, after exposure, held in plastic holding cups and provided 10% glucose for 72 h to allow enough time to become gravid. Prior to dissection, mosquitoes were killed by placing them in a freezer at −20 °C for 5–10 min and then dissected on a dissecting slide by separating the abdomen from the head and thorax to expose the ovaries using dissecting needles. After

dissection, eggs and ovaries of each mosquito were observed and photographed using a microscope equipped with a digital camera at 4× or 10× magnification. Developmental status of the eggs in each mosquito's ovaries was classified and validated by two scorers according to Christopher's stage of egg development [15]. Mosquitoes were classified as 'fertile' if eggs had fully developed to Christopher stage V and 'infertile' if eggs had not fully developed and remained in stages I–IV (see Figure 1).

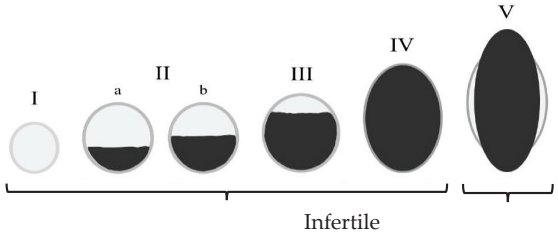

Figure 1. Christopher stages of egg development. Mosquitos whose eggs have fully developed to stage V (normal elongated, boat/sausage-shaped eggs with lateral floats) are classified as 'fecund' or 'fertile'. If eggs have not fully developed and remain in stages I–IV (less elongated, round shape, lacking floats), the mosquito is classified as 'non-fecund' or 'infertile'.

The third dataset contained 125 free-flying freshly blood-fed pyrethroid-resistant female *An. funestus* s.l. mosquitoes collected from the wall and roof of houses in Mwanza, Northwest Tanzania. Of these mosquitos, 46.4% (n = 58) were classed as being infertile and 53.6% (n = 67) were classified as being fertile. Dataset 4 also contained free-flying freshly blood-fed pyrethroid-resistant female mosquitoes collected from the wall and roof of houses in Mwanza, Northwest Tanzania. However, these were *An. gambiae* s.l., 56.8% (n = 50) classed as infertile and 43.2% (n = 38) classed as fertile. All samples from datasets 3 and 4 were, after collection and following the CDC bottle bioassay guidelines [40], immediately exposed to glass bottles treated with 1× the diagnostic dose of 100 µg/mL of PPF solution or control bottles treated with acetone for 60 min and left for 72 h post exposure to allow time to become gravid. Dissection was then carried out under a stereoscopic dissecting microscope (using a Nikon MODEL C-PSN) at 5× magnification to assess ovary development. The status of ovaries and eggs was again categorised by two scorers as either 'fertile' or 'infertile' according to Christopher's stage of egg development, with those in Christopher stage V determined to be 'fertile' and those in stages I–IV classed as 'infertile' [13]. After dissection, one image per mosquito was captured with a Motic camera microscope into a tablet PC.

2.2. Pre-Processing and Train/Test Split

After data were loaded into Python, all images were rescaled to 224 × 224 pixels to ensure consistency and improve processing times (Figure 2A). Before data were analysed, images were first randomly allocated to a training and a test set using a respective split of 70% (n = 367) and 30% (n = 157). A training set is used to teach a model to classify the correct domain. The set used here to train the model consisted of a total of 367 images, 151 (41.1%) classed as fertile and 216 (58.9%) classed as infertile. The test set is used to measure the accuracy of a model. Here, 157 total images were allocated to testing accuracy, with 76 (48.4%) classed as fertile and 81 (51.6%) classed as infertile.

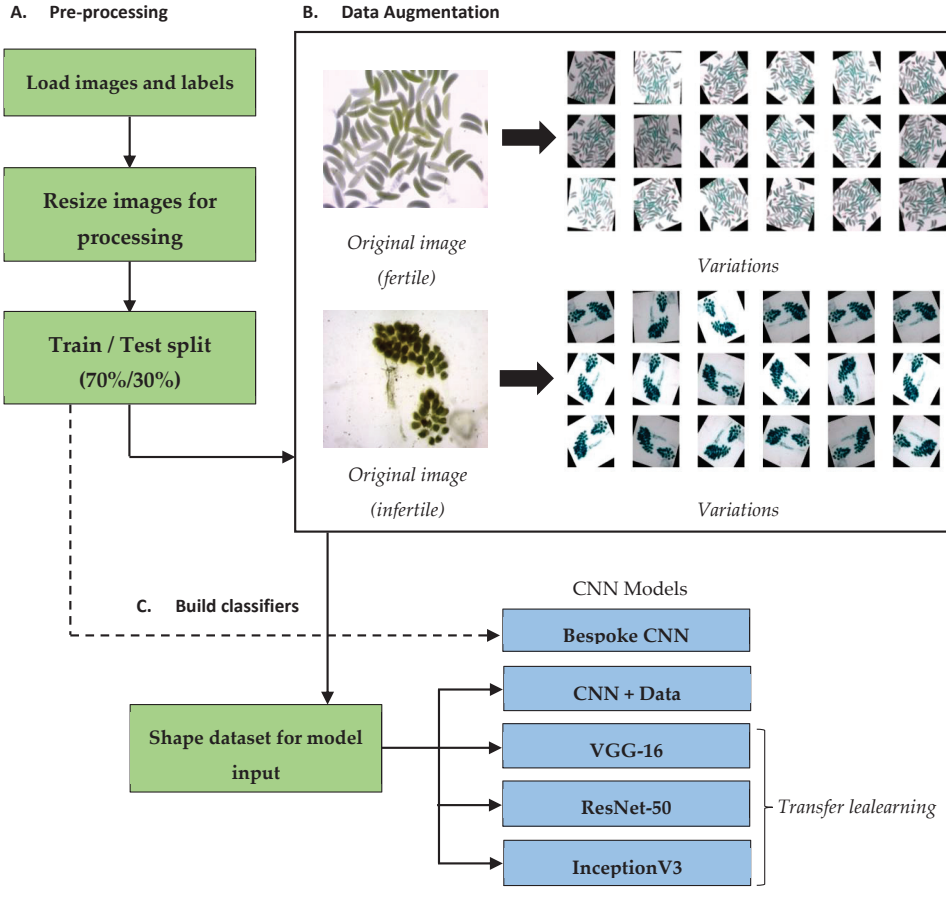

Figure 2. Summary of analysis workflow. (**A**) Data are pre-processed as described in Section 2.2. Images and labels are loaded before the images are resized and undergo a random 70%/30% split into training and test sets. (**B**) The training set undergoes data augmentation as described in Section 2.3. Each original image produces 18 variations based on a random rotation around 360°, a random horizontal flip, a random vertical flip, and a random brightness shift between 60% and 140%. Each variation retains the same classification as its original. (**C**) The training set is fitted to a range of CNNs, and classifiers are built and tested as described in Section 2.4.

2.3. Data Augmentation

To overcome the limited dataset, data augmentation was employed to increase the number of images available for training. A total of 18 random transformations were applied to each image in the training set. The original images were retained, and each variant maintained its original's classification label ('fertile' or 'infertile'). Each variant underwent a random transformation along four dimensions: (1) a random rotation around 360°; (2) a randomised horizontal flip; (3) a randomised vertical flip; (4) a random brightness shift between 0.6 and 1.4. See Figure 2B for examples of this data augmentation on a fertile and an infertile ovary image. When images are rotated, a void is created around the edges. These voids can be filled by a number of means (e.g., by repeating the whole image or the neighbouring pixel). Experimentation found that leaving these voids black had the least impact on classification. Data augmentation was only applied to the training set, increasing it from 367 to 6973 images. The test set did not undergo any data augmentation.

2.4. Analysis

To prepare data for processing by the CNNs, all images were resized (Figure 2C). The dimensions of each image were rescaled to the correct input shape for the training algorithm (224 × 224 pixels for the bespoke CNN, VGG-16, and ResNet-50 and 229 × 229 for InceptionV3). Resizing images in this manner also ensures that the magnification, resolution, or quality of the photos available when using the tool do not affect classification.

Before transfer learning was undertaken, a benchmark was established using a bespoke handmade CNN in TensorFlow. The architecture used for this CNN comprised a ReLU activated 3 × 3 input layer with 16 nodes, a (1, 1) stride, and 'same' padding (so that output size was equal to input size). This input layer then fed into three 3 × 3 ReLU activated hidden layers, with the same stride and padding as the input layer and whose number of nodes doubled from the previous layer (e.g., 16, 32, 64, and 128). Each convolutional layer fed into a 2 × 2 pooling layer, with a (2, 2) stride, to prevent overfitting. The final hidden layer was used as the input into a binary densely connected softmax output layer to capture either fertile (0) or infertile (1). As the model was a binary classifier, it was compiled using the 'Sparse Categorical Cross-Entropy' cost method, 'Root-Mean-Squared Propagation' optimiser, and 'Binary Accuracy' metric [41]. The model was trained against two training sets and used to generate two classifiers. The first classifier was trained against the original, pre-data augmentation, training set (i.e., 367 images) and the second full training set including data augmentation (i.e., 6973 images). During fitting, experimentation found that five epochs and a batch size of 32 produced the optimal performance. These models provided two benchmarks showing the impact of both data augmentation and transfer learning.

Once a benchmark was established, transfer learning was undertaken. The VGG-16 [26], ResNet-50 [27], and InceptionV3 [29] architectures with parameters pretrained against the ImageNet dataset were repurposed using the full training set (i.e., 6973 images). Although the architectures' layers were frozen, to maintain their ImageNet weighting, each was slightly altered for its new purpose. The output layer of each architecture was replaced with a densely connected softmax layer with two outputs, so as to accommodate the binary classification of 'fertile' or 'infertile'. These altered models were then compiled and fit to the training set. As each model is a deep net classifying a binary problem, all three were compiled using the 'Sparse Categorical Cross-Entropy' cost method, 'adam' optimiser, and 'Binary Accuracy' metric [41]. The training data were then used to improve the target predictive function of the architectures to detect and classify fertility status. When fitting, manual fine-tuning of the models' hyperparameters found that five epochs and a batch size of 32 maximised performance.

2.5. Resources and Requirements

Image pre-processing, data augmentation, and analysis were performed using the TensorFlow 2.4.1 library in Python through a Jupyter notebook created for this project by the lead author. All analysis found here was performed on an Intel 2.20 GHz 10 Core Xeon Silver 4114 CPU equipped on a desktop computer with 25.8 GB of RAM.

3. Results

For this study, one bespoke CNN architecture was created to classify data, and transfer learning was used to repurpose the existing VGG-16, ResNet-50, and InceptionV3 architectures. All architectures except one were retrained using the augmented training set (e.g., 6973 images). A version of the bespoke CNN was trained against the original, pre-data augmentation training set (e.g., 367 images) for benchmarking. The accuracy of all models was then measured against the test set (157 images). For a summary of the model performance, see Table 2.

Table 2. Performance of transfer learning architectures against the test set.

Architecture	Accuracy [1]	Recall (Fer) [2]	Recall (Inf) [3]	Precision (Fer) [4]	Precision (Inf) [5]	Speed [6]
Bespoke CNN	0.777	0.951	0.592	0.918	0.713	28.1 s
CNN + data Augmentation	0.815	0.901	0.724	0.873	0.777	28.7 s
VGG-16	0.885	0.901	0.868	0.892	0.880	41.7 s
ResNet-50	0.943	0.951	0.934	0.947	0.939	38.5 s
InceptionV3	0.803	0.716	0.895	0.747	0.879	36.5 s

[1] Accuracy—correct predictions divided by total number of predictions; [2] Recall (Fer)—fraction of fertile observations successfully retrieved; [3] Recall (Inf)—fraction of infertile observations successfully retrieved; [4] Precision (Fer)—true fertile predictions divided by total fertile predictions; [5] Precision (Inf)—true infertile predictions divided by total infertile predictions; [6] Speed—mean time (in seconds) over five repetitions for the model to load and classify 157 images.

3.1. Classification Accuracy

Accuracy was measured by comparing a model's classification of the images within the test set against that of the human experts, with a final accuracy rate calculated by dividing the number of correct predictions of 'fertile' or 'infertile' by the number of total predictions. Recall and precision were measured to ensure there was no imbalance in the accuracy of classes. The bespoke architecture achieved a benchmark of 78% without data augmentation but had significant skew toward 'fertile' predictions. Accuracy increased to 82%, with less skew toward 'fertile', when the augmented training set was used. When transfer learning was employed, the VGG-16 architecture achieved an accuracy of 89% with a satisfactory balance between classification, and the InceptionV3 architecture reached an accuracy similar to the benchmark of 80%, but with slight skew toward 'infertile.' However, the ResNet-50 architecture was able to attain the highest performance of all architectures, scoring an accuracy of 94% with a good balance between classes when measured against the test set. This is a level of precision close to the human accuracy rate of 99%. For a confusion matrix detailing the ResNet-50 architecture's performance, in this instance, see Table 3. As the images used to train and test the were all pre-processed, the magnification, resolution, or quality of image should not affect classification, and the accuracy scores reported here should be representative of real-world use.

Table 3. Confusion matrix for ResNet-50.

n = 157 [1]	Predicted Infertile	Predicted Fertile	
Actual Infertile	77	4	81
Actual Fertile	5	71	76
	82	75	

[1] Confusion matrix—comparison of actual values with those predicted by the model and giving details on true positive (top-left), true negative (bottom-right), false positive (top-right), and false negative (bottom-left) rates.

3.2. Classification Speed

All models were able to classify the full test set in under 1 min. To import all the necessary Python libraries, build the architecture, load the architecture with the pretrained fertility classification weights, and get the model's fertility prediction for the 157 images in the test set took a mean time (over five repetitions) of 28 s for the bespoke architecture, 41.7 s using the VGG-16 architecture, 38.5 s for ResNet-50, and 36.5 s for InceptionV3. This compares with an estimated 5 min 14 s taken for one human expert to classify the same number of images (figure determined by multiplying 157 by the mean time of 2 s taken for four trained technicians to classify 30 random ovary images).

4. Discussion

This study aimed to use deep learning, data augmentation, and transfer learning to develop an automatic method for the classification of mosquito fecundity. It was determined that, for a solution to this problem to be appropriate, it must (1) require no, or limited, expert knowledge to categorise an image, (2) achieve close to the human accuracy

rate of 99–100%, (3) be in an easily distributable, non-proprietary, and low-cost format, and (4) classify an image faster than the estimated 2 s taken by human experts.

As such, we propose that a ResNet-50 CNN architecture [27], trained against the ImageNet database, be repurposed and fine-tuned to classify the fertility status ('fertile' or 'infertile') of *Anopheles* mosquito ovaries. Classification was based on Christopher's stages of egg development [15], with eggs in stage V classed as 'fertile' and those eggs remaining in stages I–IV labelled as 'infertile.' Here, we show that such a model is capable of automatically classifying 157 images with a 94% accuracy rate in less than 40 s. Furthermore, as the model is built using TensorFlow 2.4.1, it uses a freely available, accessible, and robust opensource technology that is easily distributable via the web or mobile phones [41]. Consequently, the approach detailed in this study meets three of its aims, as it does not require any experts to categorise an image, it is easily distributable in a free format, and it can classify images faster than an expert. However, although the accuracy rate of the model does not achieve that of a human expert, it is still highly precise and is only 5% less accurate than trained experts. Furthermore, it is likely that this accuracy rate of 94% can be raised as more data become available.

Such a model is useful when assessing the efficacy of PPF-based tools through measurements of induced sterility in laboratory reared and field-collected populations of mosquitoes [12], and it could be particularly useful for large-volume bioassays done for durability monitoring of bio-efficacy of PPF-treated ITNs distributed in disease-endemic communities over time. It can also be used in bioassays performed during resistance monitoring, whereby field-collected females are exposed to a discriminating concentration of PPF to measure induced sterility [8]. This is a practical and accessible tool available to all researchers studying the efficacy of PPF or other insecticides with a similar mode of action.

Although offering several advancements over the existing manual method for classifying ovary status via dissection and examination, the model presented here is subject to its own limitations. Machine learning will not remove the need for trained technicians to dissect ovaries, only the assessment of their fertility status. Consequently, some equipment and expertise to dissect samples and to take digital colour images are still required to use the model. However, as taking photos of dissected ovaries is standard practice for record keeping and quality control, this model's need for images should not add additional work but increase objectivity and reproducibility while removing the need for a second trained technician to confirm classification. A second limitation to the current model comes from the dataset included in its training. As only pyrethroid-resistant *Anopheles* mosquito ovaries exposed to PPF were included in this study, its results are not generalisable to other species, arthropods, or insecticides. Thirdly, as there are no established dissection and imaging guidelines for capturing mosquito ovaries, there may be considerable divergence between the methods and tools employed at different sites. This may mean that the model is currently only generalisable to those locations that use techniques similar to those detailed in this paper's methods. However, the scale of this divergence, if any, is not currently known. Lastly, although a distributable application of the ResNet-50 model is currently in development, a version of the tool accessible via the internet is not yet available. Consequently, some knowledge of Python is currently necessary to employ the classifier.

It is likely that developments can be made to improve performance and accessibility. For example, to increase accuracy and applicability of the classification tool, the training set could be expanded to include samples exposed to other growth regulators or insecticides of interest, images from a broader range of sites, or other species of mosquito (including all cryptic subspecies of the *An. gambiae* complex). Additionally, accuracy and generalisability may be increased through the use of a fuzzy image classifier or classification using fuzzy logic, rather than a CNN. This alternative approach may improve precision as it could account for any ambiguity in the image dataset [42]. Furthermore, as the current model is limited to a binary classification of 'fertile' or 'infertile', it could be developed to capture the five Christopher stages of egg development or count the number of eggs in the dissected ovaries. Moreover, although the use of images from multiple locations in this study should

ensure that the model is robust enough to deal with differences in the dissection and imaging of samples, standard operating procedures concerning dissection and imaging need be developed to support the use of the classification tool. Lastly, the ResNet-50 model is currently only available via a Jupyter notebook; however, as a version of the model that can be accessed via the web is in development, a free and easy-to-use version of the model could be made freely available.

5. Conclusions

In conclusion, the reliance on manual scoring of mosquito egg development to determine the impact of PPF on the fertility of *Anopheles* mosquitos requires a level of expertise and experience that is not always available. This paper shows these limitations can be overcome using a ResNet-50 CNN model that automates the classification of egg development as a measure of fertility status. Such a model is fast, can achieve an acceptable level of precision, is in a robust format, and has the potential to be easily distributed in a practical, accessible, and freely available manner. Furthermore, this approach is applicable to scoring the fertility of mosquitoes exposed to any PPF-treated tool or similarly acting insecticide or insect growth regulator, which causes the same impact on ovary morphology, as well as applicable during bioassays performed to measure efficacy of these tools and in resistance monitoring.

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Conflicts of Interest: The authors declare no conflict of interest.

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A Practical Insecticide Resistance Monitoring Bioassay for Orally Ingested Dinotefuran in Anopheles Malaria Vectors

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Simple Summary: When insecticides are used to control mosquitoes, resistance is likely to develop over time. It is important to monitor the trait so that an alternative insecticide class can be deployed if needed, to sustain the efficiency of the intervention. Most insecticides for control of adult malaria vectors are used in treated bed nets or sprayed on walls where mosquitoes rest, so that mosquitoes contact them through their tarsi (feet). To control mosquitoes which are becoming resistant to these tools, new insecticide-based tools using both different chemistry and mode of uptake have been developed. One example of these is Attractive Toxic Sugar Baits (ATSBs), from which mosquitoes feed and ingest insecticide that kills them. However, different methods may be needed to monitor for resistance against interventions that have different modes of uptake. This study employed a method for applying insecticide directly onto a mosquito and measuring mortality, and the results were related to mortality from the same insecticide when ingested. This demonstrated that the method may be suitable to detect signs of resistance developing in mosquito populations targeted with ATSBs. Application of the method in wild populations will provide further validation.

Abstract: Attractive Toxic Sugar Baits (ATSB) deployed outdoors are likely to be particularly effective against outdoor biting mosquitoes and, if they contain insecticides with a different mode of action, mosquitoes resistant to pyrethroids. One such ATSB based on the neonicotinoid dinotefuran is currently under evaluation in Africa. As with any insecticide-based intervention, it will be important to monitor for the possible emergence of vector resistance. While methods for detecting resistance to insecticides via tarsal contact are recommended by the World Health Organization (WHO), these may not be applicable for orally ingested insecticides. Here, a new ingestion assay, appropriate for a controlled laboratory setting, is described using fluorescein sodium salt (uranine) as a feeding marker. Conventional topical application bioassays, more appropriate for routine deployment, have also been used to apply dinotefuran to the thorax of adult *Anopheles* mosquitoes with an organic carrier to bypass lipid cuticle barriers. The two methods were compared by establishing lethal doses (LD) in several *Anopheles* strains. The similarity of the ratios of susceptibility to dinotefuran between pairs of pyrethroid susceptible and resistant strains validates topical application as a suitable, more practical and field applicable method for monitoring for the emergence of resistance to orally ingested dinotefuran. A discriminating dose is proposed, which will be further validated against field populations and used to routinely monitor for the emergence of resistance alongside ATSB trials.

Keywords: insecticide resistance; Attractive Toxic Sugar Bait (ATSB); Attractive Targeted Sugar Bait (ATSB); diagnostic bioassay; resistance monitoring

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1. Introduction

The prevention of vector borne diseases is often achieved by controlling the insect population, which currently largely relies on the use of insecticides. Malaria prevalence has halved since 2000, primarily due to vector control interventions, saving 660 million lives, with a large part of the reduction being attributable to the use of insecticides [1]. The primary vector control tools employed against malaria are insecticide treated nets (ITNs) and indoor residual spray (IRS). However, insecticide resistance represents a major threat to human health. Alternative interventions with different active ingredients and/or modes of action, capable of controlling insecticide resistant vectors, as well as vectors which transmit malaria outdoors, are urgently required to ensure the sustainability of malaria control interventions.

A number of Attractive Toxic Sugar Baits (ATSBs) are being evaluated as part of an Integrated Vector Management (IVM) approach. ATSBs deployed outdoors are likely to be particularly effective against outdoor biting mosquitoes, as well as mosquitoes that are resistant to pyrethroid insecticides. An ATSB has been developed by Westham Co. which utilizes the neonicotinoid dinotefuran. The bait station includes a permeable membrane that allows volatile attractive compounds to be emitted and encourage feeding by mosquitoes yet minimizes feeding by non-target organisms (NTOs) and tarsal contact of both mosquitoes and NTOs with the insecticide-treated bait. Because of the inclusion of this permeable membrane, the product is termed an Attractive *Targeted* Sugar Bait (ATSB®) [2]. These bait stations have been shown to be effective in controlling malaria vectors in Mali [3] and are under evaluation in conjunction with the Innovative Vector Control Consortium (IVCC) in trials in Zambia, Kenya, and Mali. Dinotefuran is included as the active ingredient and, since this insecticide is new to public health, it is not expected that target mosquito populations will carry any resistance to it, though another neonicotinoid, clothianidin, is now in use for public health and cross-resistance is a risk. As with any new vector control tool based on insecticides, once dinotefuran-based ATSBs are deployed, susceptibility testing will need to be introduced to allow early detection of possible emerging resistance and enable evidence-based resistance management strategies. Conventionally discriminating (or diagnostic) dose bioassays are used to detect resistance to insecticides encountered by mosquitoes through tarsal contact on an ITN or IRS, and so a WHO tube assay [4] or the Centers for Disease Control and Prevention (CDC) bottle bioassay [5] is used. Survival in a discriminating dose (DD) or discriminating concentration (DC) assay is a sign of possible resistance in the target population.

Such diagnostic bioassays are not available for orally ingested insecticides, and nor is a DC recommended by the WHO for susceptibility monitoring of dinotefuran [4] via tarsal contact. Methods for detecting resistance to neurotoxic insecticides via tarsal contact may not be applicable for the orally ingested dinotefuran due to its negative log P, which inhibits tarsal uptake due to epicuticular lipids and barriers. In addition, it is possible that this different method of exposure may be affected by different resistance mechanisms than those responsible for resistance against contact insecticides. It is therefore desirable to establish a suitable method to screen for dinotefuran resistance in ATSB deployment sites.

Ideally, mosquitoes would be fed a discriminating dose of dinotefuran ingested in a sugar solution to most closely match the exposure route in an ATSB. However, to date only a few methods, rather complicated in terms of practical implementation, have been established to feed a spiked sugar meal to mosquitoes with a high enough feeding rate to allow this form of resistance monitoring to be done [6,7]. Assays based on feeding an AI to insects in a sugar meal are vulnerable to huge variability and poor accuracy, due to poor feeding rates, especially with recently colonized or field-caught mosquitoes, and variable volumes taken up by those mosquitoes that do feed. Topical application of insecticide bypasses tarsal barriers by applying insecticide solutions in lipophilic solvent directly to the thorax of the mosquito to be taken in through the cuticle [4]. Although this is not the same entry system as the proposed delivery via ingestion, it is a technique that also bypasses cuticular barriers and therefore may be sufficiently representative of oral uptake whilst

being easily applicable for routine susceptibility monitoring in field sites. Indeed, it has been shown in agricultural pests that the response to exposure to neurotoxic insecticides by topical application is a good proxy for the response to ingestion and that resistance monitoring assays for oral insecticides can be based on topical application [8]. Topical application is relatively quick with even large numbers of mosquitoes and can be done with fairly straightforward portable equipment, and as such is a more robust method for susceptibility testing.

Here, an oral application assay has been developed, able to determine dose response curves among two *Anopheles* strains, that prevent tarsal contact while allowing ingestion of a spiked sugar meal. Practical topical application bioassays were also developed by applying dinotefuran to the thorax of adult *Anopheles* mosquitoes with organic carrier to bypass lipid cuticle barriers. The topical application dose response was compared with the oral toxicity bioassays across several *Anopheles* strains.

2. Materials and Methods

2.1. Mosquito Rearing

Mosquitoes were reared within the insectaries of Liverpool Insect Testing Establishment (LITE) at the Liverpool School of Tropical Medicine as previously described [9], at 26 ± 2 °C and 80 ± 10% relative humidity. Four strains were used for experiments, all described by Williams et al. [9]. Kisumu is an insecticide-susceptible strain of *Anopheles gambiae*, colonized from Kenya in 1975. VK7 2014 is a strain of *An. coluzzi* colonized from Valley de Kou, Burkina Faso, in 2014 and resistant to pyrethroids and Dichlorodiphenyltrichloroethane (DDT) as a result of both target site and metabolic resistance mechanisms. Fang is a susceptible colony of *An. funestus* colonized from Calueque, Southern Angola in 2015. FUMOZ-R, also *An. funestus,* was colonized from Mozambique in 2000 before being selected by exposure to lambda-cyhalothrin to produce a strain with a high level of metabolic resistance [10]. Though not of the same species, the VK7 2014 strain was compared to Kisumu, a model laboratory colony of the *Anopheles gambiae* species complex which is susceptible to all classes of insecticide. A direct species comparison of susceptible (Fang) and pyrethroid resistant (FUMOZ-R) strains was possible for *An. Funestus*.

All tests were carried out using 2–5 day old female mosquitoes which had been allowed to sugar feed and mate but not blood feed prior to testing. For mosquito size for each sample, refer to Supplementary Materials.

2.2. Ingestion Assay

Between one hundred and two hundred 2–5 day old female mosquitoes were starved in a standard (30 cm × 30 cm × 30 cm) BugDorm-1 (MegaView Science Co., Ltd., Taichung, Taiwan) cage for approximately 18 h with ad libitum access to purified (Merck Millipore, Darmstadt, Germany) water-soaked cotton on top of the cage mesh. Mosquitoes were then exposed to insecticide mixed into sugar solution by adding 2 feeders, designed to prevent tarsal contact yet allow easy feeding, to each cage for 24 h (Figure 1).

The sugar solution was 10% sucrose (granulated sugar in de-ionized water), 0.8% Uranine (Fluorescein Sodium Salt; Honeywell, Charlotte, NC, USA) fluorescent marker, and treatment-dependent insecticide concentration (0.000001%, 0.00001%, 0.00002%, 0.000025%, 0.00005%, 0.000075%, 0.0001%, and 0.001% w/v). Between 1 and 5 replications were performed per concentration and between 5 and 9 replications for each control to collect sufficient data to generate LD values using Rstudio (See Supplementary data). Technical grade (98.7%) dinotefuran was sourced from Sigma–Aldrich (Manchester, UK). 10 mL of each insecticide concentration solution was used to soak cotton wool inside sugar feeders less than 5 min prior to adding the feeder to the cage. Two feeders were used per cage to ensure mosquitoes had adequate access to sugar/insecticide solution.

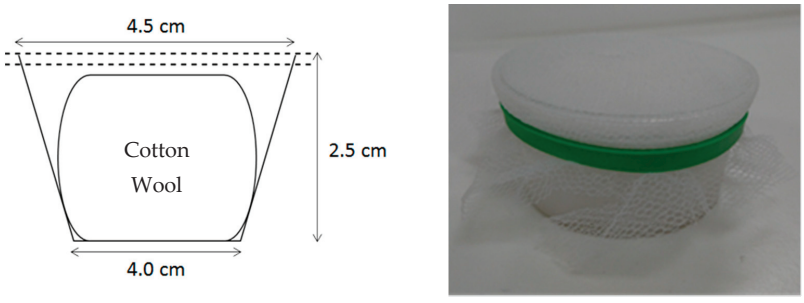


Figure 1. Sugar feeder made from a plastic pot of 2.5 cm height, large radius of 4.5 cm, and small radius of 4 cm. A wad of cotton wool was pressed into the pot to be just below the upper lip without touching the netting (judged by eye) and soaked in sugar solution into which the required concentration of insecticide was dissolved. A double layer of netting was stretched across the top and secured using an elastic band.

After the exposure period, all mosquitoes were aspirated out of each cage into holding cups, separated by treatment as well as living or dead, then frozen at −20 °C. Once mosquitoes were killed (usually 1–2 h at freezing temperatures), they were scored for fluorescence using a Leica MZ 10 F microscope (Leica Microsystems, Milton Keynes, UK) under a yellow-fluorescent protein (YFP) filter (Figure 2). Only those mosquitoes that were scored as being positive for feeding by fluorescence were included in the mortality calculations. Feeding rate was calculated for each replicate test from the proportion of fluorescent positive mosquitoes relative to all exposed mosquitoes. All raw bioassay data is available in Supplementary Materials.

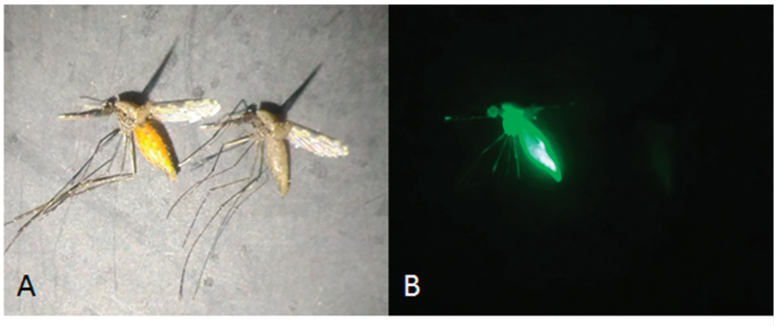


Figure 2. Side by side comparison of two adult female mosquitoes, one fed on 10% sugar solution only (**right**) and one fed on 10% sugar solution with 0.8% Uranine (**left**). Photographs are taken using white light (**A**) and UV light under a YFP filter (**B**).

2.3. Topical Application

Cohorts of 10 2–5 day old female mosquitoes at a time were knocked down using CO_2 for 20 s before being transferred to a petri dish with filter paper dampened with purified water. While knocked down, the mosquitoes were positioned ventrally so that their dorsi were easily accessible. 0.2 µL aliquots of insecticide in acetone solution were applied to the dorsal side of each mosquito thorax using a 10 µL Hamilton syringe (Scientific Laboratory Supplies Ltd. (SLS), Nottingham, UK). Mosquitoes were then transferred back into holding cups and knock down or mortality was scored at 30 min, 60 min, and 24 h post-exposure. As well as an acetone-only negative control and a positive control of Permethrin at a concentration of 0.1%, six doses of dinotefuran were applied to Kisumu (0.0002%, 0.0005%, 0.0001%, 0.0025%, 0.004%, and 0.005% w/v). These six and a further four concentrations

were applied to VK7 2014 (0.01%, 0.02%, 0.04%, and 0.1% *w*/*v*). For the *Anopheles funestus* strains, the range was reduced to three concentrations in addition to the positive and negative controls: 0.0004%, 0.004%, and 0.02% *w*/*v*. Three replicates were performed, each using different generations of each strain such that 60 Kisumu individuals were treated with each concentration of insecticide and 50 for each control. Similarly, at least three replicates totaling 60 VK7 2014 individuals were tested at each concentration. However, only 20 VK7 2014 individuals were tested at 0.1% as a part of range finding where mortality had already reached 100% in lower concentrations. For both Fang and FUMOZ-R strains, at least 90 mosquitoes were tested at each concentration over three replicates. Data sets from 24 h post exposure were used to generate values for lethal doses (LD). All raw bioassay data is available in Supplementary Materials.

2.4. Establishing Dose Response Curves

A dose response dataset was established for dinotefuran applied by topical application, as well as by sugar feeding assay in a susceptible strain of *Anopheles gambiae* (Kisumu, [9]) by applying a range of concentrations which gave mortality ranging from 0 to 100%. Topical application gives doses in nanograms per mosquito, converted to nanograms per milligram of mosquito by taking the averages of sample weights of 20 mosquitoes. For the ingestion assay, doses in nanograms per milligram of mosquito were found by estimating the average meal sizes of 10% sugar solution and 0.8% Uranine using fluorimetry (see Appendix A—Quantifying the Average Size of a Sugar Meal Using Fluorescein Sodium Salt (Uranine)). Dose was then inferred through the estimated average meal size of 0.4 μL per feed against the average mosquito mass of 20 individuals per sample.

2.5. Calculating LD Values and Resistance Ratios

LD_{50} and LD_{95} values with associated 95% confidence intervals were obtained for each strain by fitting the data to a dose response model ('drc' package [11] in R Studio [12].

Susceptibility to dinotefuran was compared between strains by calculating a resistance ratio by dividing the LD_{50} of the pyrethroid resistant strain in each species pair by the LD_{50} of the susceptible strain.

3. Results

3.1. Establishing Dose Response Curves by Ingestion Assay

An ingestion assay was used to plot dose response curves for orally ingested dinotefuran in a sugar solution. The ratio of LD_{50} values in each pair of strains, Kisumu vs. VK7 2014 and Fang vs. FUMOZ-R, were similar, so only Kisumu and VK7 2014 were selected to establish further dose response curves by the ingestion assay for comparison between the two methods.

The feeding rate between tests ranged from 70 to 98% and 80 to 97% in Kisumu and VK7 2014 cohorts, respectively, and there was no visible trend with dinotefuran concentration (see Supplementary Material). The LD_{50} for Kisumu was 0.08 (0.06–0.11) ng of dinotefuran per mg of mosquito and the value for VK7 2014 was 0.17 (0.12–0.23) ng of dinotefuran per mg of mosquito (Figure 3), resulting in a resistance ratio of 2.13. Lethal doses (LD_{50} and LD_{95}) by ingestion are shown in Table 1.

Table 1. Lethal doses and lethal concentrations of dinotefuran ingested in a sugar solution in two strains of *Anopheles* mosquitoes. 95% CI given in parentheses. Kisumu is a lab strain of *Anopheles gambiae*, VK7 2014 is *An. coluzzii*.

Strain	LD_{50}	LC_{50}	LD_{95}	LC_{95}
	ng/mg of Mosquito	ng per Mosquito	ng/mg of Mosquito	ng per Mosquito
Kisumu	0.08 (0.06–0.11)	0.12 (0.09–0.17)	0.29 (0.12–0.67)	0.45 (0.19–1.04)
VK7 2014	0.17 (0.12–0.23)	0.2 (0.15–0.28)	0.65 (0.3–1.38)	0.79 (0.37–1.69)

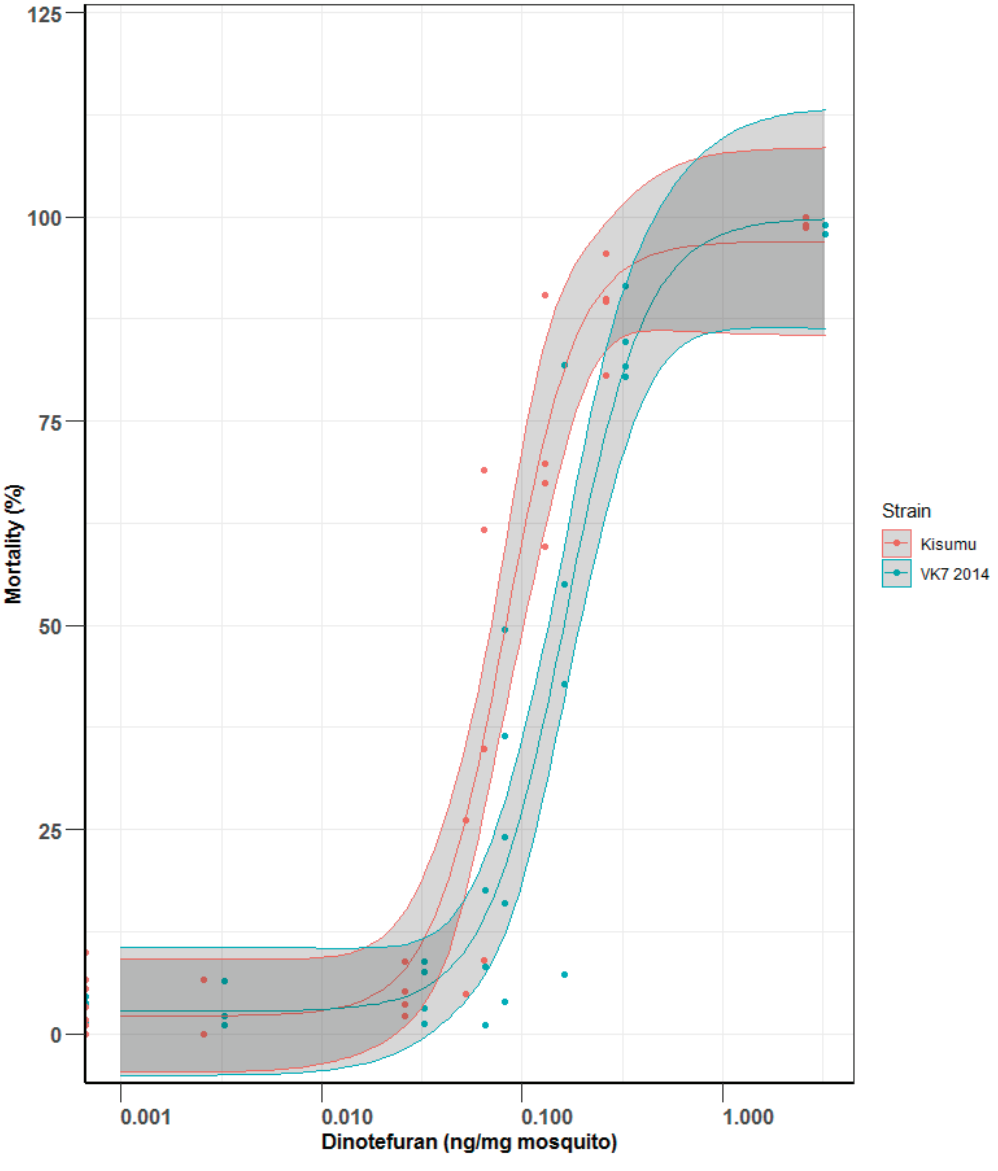
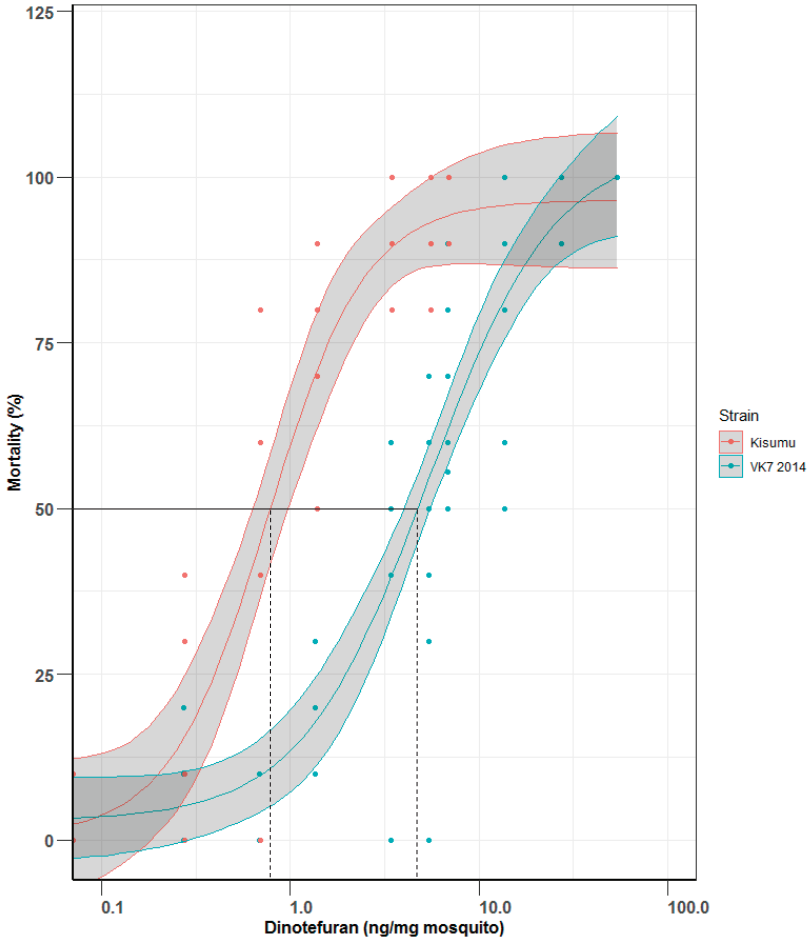


Figure 3. Mortality comparison between Kisumu and VK7 strains when fed on sugar solution spiked with dinotefuran at known concentrations, resulting in doses of dinotefuran in nanograms per milligram of mosquito. Central lines of each curve represent the dose response of each species. Black lines indicate LD_{50} values; red refers to the mortality curve of Kisumu; blue refers to the mortality curve of VK7 2014. The shaded areas of each curve represent 95% CI values, generated by R software using the ggplot2 package [13].

3.2. Establishing Dose Response Curves by Topical Application

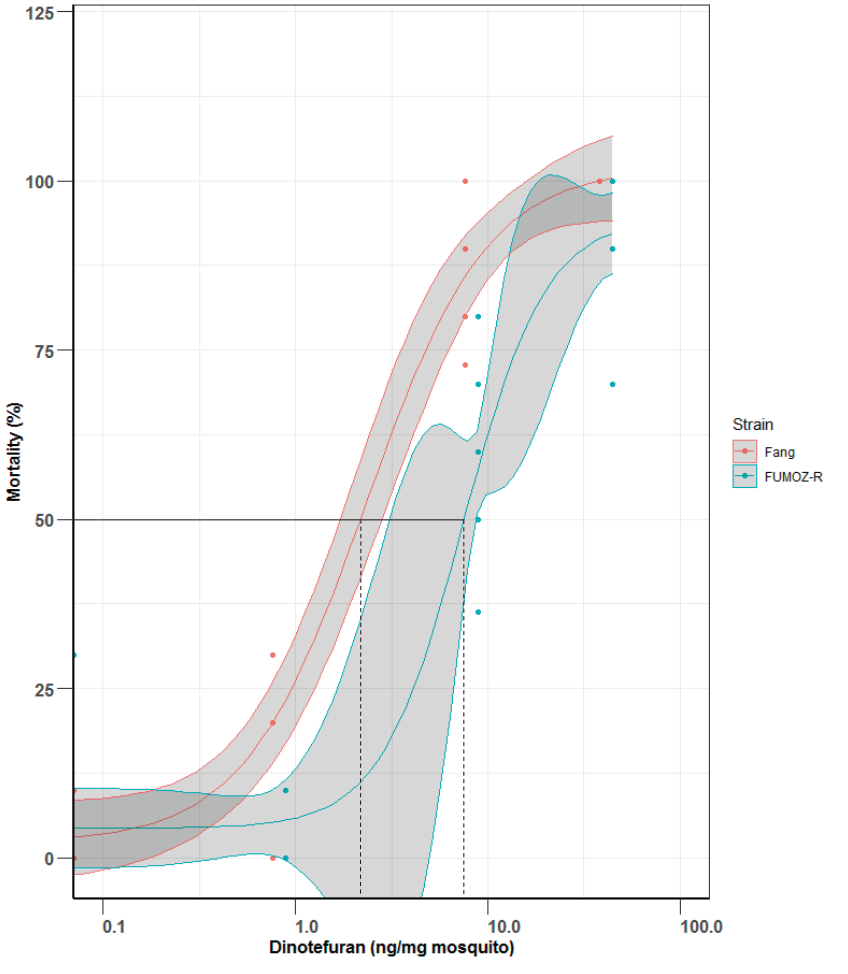
Because of the practical challenges in performing ingestion assays, particularly in field settings and at high throughput, dose response curves were also plotted using topically applied dinotefuran in two pairs of *Anopheles* strains as a comparator to the ingestion assay.

Topical bioassays (Figure 4A,B) for Kisumu generated an LD_{50} value of 0.75 (0.55–1.03) ng of dinotefuran per mg of mosquito and VK7 2014 assays generated an LD_{50} value of 5.34 (3.97–7.19) ng of dinotefuran per mg of mosquito; together this gives a resistance ratio of 7.12. LD_{50} values for Fang and FUMOZ-R were 2.31 (1.63–3.27) and 7.47 (5.98–9.32) ng of dinotefuran per mg of mosquito, respectively, resulting in a resistance ratio of 3.23. Lethal doses (LD_{50} and LD_{95}) by topical application are shown in Table 2.



(**A**)

Figure 4. *Cont.*



(**B**)

Figure 4. Mortality comparisons between (**A**) Kisumu and VK7 2014 strains and (**B**) Fang and FUMOZ-R strains in a topical application bioassay. Central lines of each curve represent the dose response of each species. Black lines indicate LD_{50} values on both graphs; red lines show mortality curves of the insecticide susceptible strains of each pair of strains (Kisumu and Fang); blue lines show data for insecticide-resistant strains (VK7 2014 and FUMOZ-R). Shaded areas of each curve represent 95% CI values, generated by R software using the ggplot2 package [13]. (**A**) omits data for VK7 2014 treated with 136 ng per mg of mosquito, this gave 100% mortality with no variance as did the highest represented range (54 ng per mg of mosquito, refer to Supplementary Material) and so was removed for clarity.

Table 2. Lethal doses and lethal concentrations in four strains of *Anopheles* mosquitoes by topical application of dinotefuran. 95% CI given in parentheses. Kisumu is a lab strain of *Anopheles gambiae*, VK7 2014 is *An. coluzzii*, and Fang and FUMOZ-R are *An. funestus*.

Strain	LD_{50}	LC_{50}	LD_{95}	LC_{95}
	ng/mg of Mosquito	ng per Mosquito	ng/mg of Mosquito	ng per Mosquito
Kisumu	0.75 (0.55–1.03)	1.09 (0.80–1.49)	4.41 (1.78–10.93)	6.38 (2.57–15.82)
VK7 2014	5.34 (3.97–7.19)	7.85 (5.84–10.57)	52.35 (18.79–145.86)	76.96 (27.62–214.41)
Fang	2.31 (1.63–3.27)	2.43 (1.72–3.43)	19.64 (9.28–41.57)	20.62 (9.74–43.65)
FUMOZ-R	7.47 (5.98–9.32)	6.72 (5.38–8.39)	31.82 (3.69–274.05)	28.64 (3.33–246.65)

4. Discussion

There is a growing array of vector control tools based on insecticides which act via a range of different exposure routes. The Attractive Targeted Sugar Bait (ATSB) is the only one that involves ingestion by adult mosquitoes. One ATSB currently under evaluation includes dinotefuran, which mosquitoes feed on in a sugar-based bait. As with any insecticide-based intervention there is a need to monitor for the emergence of resistance in the target population, which conventionally has been done using testing methods which expose field caught mosquitoes of the target population via tarsal contact to a treated bottle [5] or filter paper [4]. Because the exposure route of an ingested insecticide is different to a contact insecticide, the results of these tests may not be an accurate indicator of resistance and risk of failure of ATSBs.

This study considered two alternative methods to screen for resistance. The first was an oral ingestion assay developed to prevent tarsal contact while allowing ingestion of a spiked sugar meal, the most direct test for resistance to an oral insecticide. The assay was demonstrated to be robust and quantitative enough to be able to establish a dose response in laboratory strains of *Anopheles*, including in two strains that are highly resistant to pyrethroids. By using a uranine marker, individual mosquitoes that fed were identified, and in the controlled laboratory setting of these experiments the feeding rate was high. However, the applicability of the sugar feeding assay used here in the field is limited due to the large variation of sugar feeding behavior—and thus insecticide uptake—when applied to field caught mosquitoes and using a less controlled laboratory environment. It is likely that the feeding rate, which was high in laboratory strains adapted to feeding on an artificial sugar source, would be much lower in field caught adults or adults emerging from field collected larvae. A low feeding rate would further increase the resources required to produce significant data which could be relied upon in a screen for emerging resistance. Even in these experiments conducted in tightly controlled laboratory conditions and with mosquitoes reared using standardized protocols [9], the results were varied. The methodology would be difficult to standardize sufficiently that it could be performed in multiple field sites, likely with less controlled environments, and achieve robust and comparable data. The ingestion bioassay method also requires greater resources in terms of space and time than topical application, and access to a fluorescent microscope.

The consistently high feeding rate across treatments in this study, which was not correlated with concentration of dinotefuran, suggests a lack of any detectable repellent effect of the dinotefuran. However, in adapting this method for other insecticides, there is a risk of a repellent effect reducing the feeding rate. To avoid this as a possible confounding factor, it is important to use some methods to eliminate individuals that do not feed from mortality scoring, either including uranine and scoring fluorescence as done here or using an alternative such as Trypan blue dye [6,7]. Another possible confounding factor is that it has not been established exactly how long mosquitoes may survive after ingesting particularly lower concentrations of insecticide without direct observation for the whole exposure period. It is possible mosquitoes may have fed just before collection and be scored as survivors when they may have died even minutes later. However, the assay still demonstrated sufficient sensitivity to measure a difference in mortality between

concentrations in a dose responsive manner, so this does not appear to prevent the ingestion assay being applicable for this purpose.

Because of the logistical challenges of the ingestion assay, a practical and well established topical application bioassay [14] was also used, applying dinotefuran to the thorax of adult *Anopheles* mosquitoes with organic carrier to bypass lipid cuticle barriers. The direct application of insecticide to the mosquito thorax bypasses the need for uptake of insecticide from a surface and penetration of the insecticide through the cuticle, and mortality as a result of this exposure route has been shown in other insects to correlate well with oral toxicity [8]. The variability of the data between replicates is less with topical application because parameters which define the dose taken up by the mosquito are less variable than for the ingestion assay, producing more robust data.

This study compared the topical application dose response with the respective response of the oral toxicity bioassays, across four *Anopheles* strains. The dose response curves plotted for the same strains were very similar, and there was similar relative susceptibility between the two strains tested with both methods. Topical application is a well-established method and relatively easy to apply [15]. The similarity of results between the two methods demonstrated here suggests that a topical application-based DC, determined based on WHO guidelines, could be used as a proxy for monitoring the development of resistance in field populations to orally ingested dinotefuran from ATSB stations. However, tissue-specific resistance mechanisms are not well studied and there is risk that topical application will not pick up on the emergence of an as yet unidentified ingestion specific mechanism. If potential resistance is observed in results of susceptibility monitoring using topical application, further investigation would be warranted, including exploring such possible mechanisms using ingestion assays in the laboratory. Similarly, the results of topical testing could be affected by the presence of cuticular resistance, through cuticular thickening, altered cuticle composition, or alterations in receptors that affect uptake and penetration of active ingredients. Such resistance mechanisms may be primarily overexpressed in the tissues of the mosquito that are typically in contact with insecticides, such as the tarsi. The direct application of acetone to the thorax (the solvent used to deliver insecticides during topical application) is believed to bypass these mechanisms, and no correlation between cuticular resistance and reduced mortality by topical application has yet been reported. The WHO approach to establishing a DC is to perform dose response experiments and establish LC values for a range of strains susceptible to the insecticide being tested, and then to select the highest DC established for the least susceptible strain, based either on the calculated LC values (DC = 2 × LC_{99}) or an observed LC_{100}, defined as the lowest concentration tested which reliably produces 100% mortality in susceptible strains [4]. A pragmatic decision may be made as to whether to recommend a specific DC for each species or to select the highest DC to use for a group of species, and sometimes rounding the calculated DC to a value more easily applied in field testing [16]. Based on the dose response observed and LC values calculated in this study, a DC of 100 ng/mosquito would be recommended for topically applied dinotefuran for *An. gambiae* and *An. funestus*. However, a lower tentative DC of incipient resistance, at 10 ng/mosquito, is highly recommended as well, to collect baseline susceptibility data and capture possible variation in bioassay responses among populations in the ATSB trial sites. These trials will further validate the methodology, as well as define the most appropriate DC for screening field *Anopheles* populations.

The relative susceptibility of Kisumu and VK7 2014 strains was measured by each method by calculating a resistance ratio of 5.4 by ingestion and 1.8 by topical application. Both these ratios are very low, all below 10, and do not indicate that there is resistance, but rather inherent variability in susceptibility between strains. A more robust validation of the correlation of results between the two methods by repeating this study with a strain known to be resistant to dinotefuran, or neonicotinoids in general, would help to confirm comparability of results from the two methods but to date no such resistance has been reported in field caught mosquitoes and so no such laboratory strain is available.

Another possibility would be to make use of transgenic strains which have resistance to neonicotinoids induced, using a method such as CRISPR/Cas9 [17,18].

The lethal dose of dinotefuran was lower by ingestion than by topical application (approximately 20 times). This points towards a higher toxicity when ingested, though there are several sources of variability in calculating the precise dose of dinotefuran ingested in the sugar feeding assay that mean a direct comparison cannot be made. These include different meal sizes taken by individual mosquitoes, related to body size and previous handling, some individuals taking full sugar meals and some only partial feeds, and the possibility that different volumes are ingested in treatments where insecticide is added.

The volume of bait ingested from an ATSB may be different to the volume of sugar water ingested in this assay, and the size of sugar meal may differ between mosquito populations. It is not, therefore, possible to directly compare the toxic ingested dose of dinotefuran in this assay with the actual dose of dinotefuran in the ATSBs and predict efficacy against target mosquitoes. We can, however, make some estimations based on the assumption that a similar volume of bait is ingested from an ATSB station by wild mosquitoes. The Westham ATSB stations currently under evaluation contain 0.1% dinotefuran, so a mosquito taking up 0.4 µL of bait (Appendix A) will ingest 400 ng of insecticide, 300 times the dose shown to kill 100% of mosquitoes in the ingestion assay. This means that if only 0.0013 µL of bait is ingested, it will be lethal to the mosquitoes. Based on the calculated LC_{50}, an amount consumed 3000 times lower than the typical sugar meal would be sufficient to kill 50% of the mosquitoes which feed on it. The bait stations should continue to be effective in populations even where resistance is seen to have emerged through monitoring, using the relatively sensitive DC which has been established.

Practically, no cross-resistance between dinotefuran and pyrethroids was observed. The pyrethroid resistance ratio for these same strains tested with permethrin previously were 145.77 (149–397) via topical application and 128.23 (81.4–198.5) via a tarsal assay [9], but the response of the same strains to dinotefuran was essentially not different or indicated a very low cross-resistance. These data confirm the utility of chemicals belonging to different Insecticide Resistance Action Committee (IRAC) mode of action (MoA) classes [19] (i.e., different target sites and/or routes of uptake for insecticide resistance management (IRM)). Neonicotinoids are nicotinic acetylcholine receptor competitive modulators (IRAC class 4A), with a high selectivity in binding to insect nicotinic acetylcholine receptor sites over that of mammal receptors [20]. The target of action is thus different to pyrethroids, which are sodium channel modulators (IRAC class 3A). None of the WHO Prequalified Vector Control Products contain dinotefuran, and so it is unlikely that mosquitoes have been exposed and developed resistance to dinotefuran. Fludora® Fusion and SumiShield 50WG IRS formulations contain clothianidin, also a neonicotinoid, and their potential for use against pyrethroid resistant insects has been demonstrated [21,22]. Extensive experiments with a proposed diagnostic concentration of 2% *w*/*v* clothianidin on filter papers failed to find conclusive evidence of resistance in 43 sites in sub-Saharan Africa [23], or in *Anopheles arabiensis* in Ethiopia [24]. No evidence of resistance to clothianidin was found in western Kenya using a DC of 150 µg/bottle [25]. However, once an insecticide is being deployed a selection pressure is applied and there is a risk of resistance evolving, and so once validated, the DC should be used to perform regular resistance monitoring in all sites where ATSBs are deployed. Validation of this methodology should also be carried out for any future insecticides used in new ATSB designs. There is also a risk of cross-resistance to dinotefuran as a result of exposure to other neonicotinoids used for vector control or in agriculture.

5. Conclusions

An approach has been demonstrated by this study for establishing a suitable method for screening for resistance to a non-contact insecticide.

A discriminating, or diagnostic concentration for topically applied dinotefuran, has been proposed and should now be validated against field mosquito populations where

ATSBs are under evaluation. Validation with other ingested insecticides is recommended as further ATSB or similar products are developed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13040311/s1, File S1: All raw data.

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Appendix A. Quantifying the Average Size of a Sugar Meal Using Fluorescein Sodium Salt (Uranine)

Appendix A.1. Introduction

To provide an estimate on the dose of insecticide taken up by mosquitoes to insecticide in ingestion assays, it is important to know the approximate size of a sugar meal taken in an environment comparative to the ingestion assays.

Appendix A.2. Methods

Mosquitoes were reared within the insectaries of LITE at the Liverpool School of Tropical Medicine at 26 ± 2 °C and 80 ± 10% relative humidity. A cohort of approximately 100 2–5 day old female *Anopheles gambiae* (Kisumu) were starved in a standard (30 × 30 × 30 cm) BugDorm-1 (MegaView Science Co., Ltd., Taichung, Taiwan) cage for approximately 18 h with ad libitum access to purified (Merck Millipore, Darmstadt, Germany) water-soaked cotton placed on the ceiling mesh of the cage, but with sugar feeders removed. Just before testing began, 20 mosquitoes were sampled at random, knocked down by CO_2 gas exposure, weighed, and then frozen. The remainder in the cage were then offered a sugar meal in pots of 10% sugar-soaked cotton with 0.8% Uranine covered in a double-layer of netting to prevent tarsal contact.

Mosquitoes were observed directly for a period of 3 h. Feeding behavior was defined as a mosquito landing on the feeder, probing, and subsequently positioning the proboscis down through the mesh and remaining still while the abdomen was observed to visibly expand, filling with some amount of the dyed sugar solution. Individuals seen to engage in feeding behavior were removed and frozen immediately after feeding ended to prevent or slow any digestive processes that could affect the fluorescence levels of the ingested sugar solution. 38 mosquitoes were observed to feed within the time period and collected in this fashion. Fed mosquitoes were then homogenized in 10 mL purified water, 2 mL of which was decanted into plastic cuvettes and read in a Trilogy™ Fluorometer (model 7200-0000, Turner Designs, San Jose, CA, USA) to give raw fluorescence units (RFU) of each solution. This was compared to a calibration curve generated using serial dilutions of the same stock of 0.8% Uranine and 10% sugar solution (Figure A1) to give the concentration of each homogenized sample. From this, the volume of sugar solution ingested by each mosquito was calculated using a simple concentration calculation (C1V1 = C2V2). Four of the twenty mosquitoes removed previously for weighing were randomly selected and were treated in the same way to control for autofluorescence or contamination.

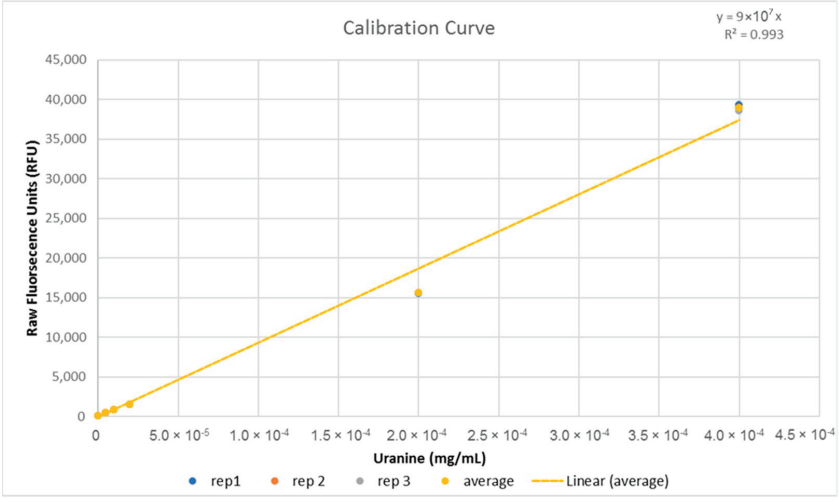

Figure A1. Calibration curve generated using a stock of 0.8% Uranine (8 mg/mL) in 10% sugar solution diluted into purified water and serially diluted to a suitable range. 2 mL was decanted and read from 10 mL of each concentration three times in the fluorometer to give raw fluorescence units and the average was used to generate the line of best fit. The equation and R^2 value of the curve is displayed on the upper right corner of the graph.

Appendix A.3. Results

The meal sizes for the 38 mosquitoes observed to feed ranged from 0.2 µL to 0.7 µL. The mean meal size was 0.4 µL (95% CI 0.35–0.43). This value was used to calculate the dose of dinotefuran ingested per mosquito in subsequent assays.

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Article

Developing Consensus Standard Operating Procedures (SOPs) to Evaluate New Types of Insecticide-Treated Nets

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Simple Summary: Malaria control relies on insecticide-based tools which target the mosquito vector. Predominantly, a group of insecticides called pyrethroids are used in these tools. Globally, however, mosquitoes are increasingly developing resistance to pyrethroids. Subsequently, new products, such as insecticide-treated nets (ITNs), which contain combinations of insecticides from different classes, or chemicals that work synergistically with pyrethroids, are being developed. Several of these new net types are being rolled out for testing and use. However, standardized methods to measure how long these nets remain active against mosquitoes are lacking, which makes evaluating the long-term efficacy of these products challenging. In this publication, we propose a pipeline used to collate and interrogate several different methods to produce a singular 'consensus standard operating procedure (SOP)', for monitoring the residual efficacy of three new net types: pyrethroid + piperonyl butoxide (PBO), pyrethroid + pyriproxyfen (PPF), and pyrethroid + chlorfenapyr (CFP).

Abstract: In response to growing concerns over the sustained effectiveness of pyrethroid-only based control tools, new products are being developed and evaluated. Some examples of these are dual-active ingredient (AI) insecticide-treated nets (ITNs) which contain secondary insecticides, or synergist ITNs which contain insecticide synergist, both in combination with a pyrethroid. These net types are often termed 'next-generation' insecticide-treated nets. Several of these new types of ITNs are being evaluated in large-scale randomized control trials (RCTs) and pilot deployment schemes at a country level. However, no methods for measuring the biological durability of the AIs or synergists on these products are currently recommended. In this publication, we describe a pipeline used to collate and interrogate several different methods to produce a singular 'consensus standard operating procedure (SOP)', for monitoring the biological durability of three new types of ITNs: pyrethroid + piperonyl butoxide (PBO), pyrethroid + pyriproxyfen (PPF), and pyrethroid + chlorfenapyr (CFP).

This process, convened under the auspices of the Innovation to Impact programme, sought to align methodologies used for conducting durability monitoring activities of next-generation ITNs.

Keywords: insecticide-treated net (ITN); PBO ITN; synergist ITN; dual-AI ITN; insecticide resistance management (IRM); method validation; durability monitoring

1. Introduction

Globally, malaria control progress is plateauing, and, in some instances, case numbers are rising [1]. Although the reasons for this are multifaceted, an increasing and intense resistance to pyrethroids in *Anopheles* vectors is almost certainly a contributing factor. Insecticide-treated nets (ITNs) have significantly contributed to the control of malaria over the past two decades [2]. However, currently, all WHO-prequalified ITNs contain pyrethroids [3], and pyrethroid resistance is widespread in all major malaria vectors [4,5].

In response to growing concerns over the sustained effectiveness of solely pyrethroid-based control tools, new products are being developed and evaluated. Examples of these are dual-active ingredient (AI) ITNs containing an additional insecticide, or synergist ITNs which contain an insecticide synergist, in combination with a pyrethroid. These net types are often termed 'next-generation' insecticide-treated nets. The second AIs have a different mode of action (MoA) from their partner pyrethroid, to improve the control of resistant vector populations.

The current methods for measuring ITN durability [6] were developed for pyrethroid-only nets, which cause rapid knockdown and death in susceptible mosquitoes. Consequently, the different MoAs of the new insecticides necessitate the need for new protocols to reliably measure net durability. In nets with the synergist piperonyl butoxide (PBO), the PBO works by improving the efficacy of the pyrethroid it is paired with, in populations with pyrethroid resistance due to increases in oxidase activity, and is itself generally non-insecticidal. Without suitable mosquito strains or net controls, it is difficult to determine if the synergist component of the net is long-lasting using the currently recommended methods. For other AIs, such as chlorfeniapyr, which targets the insect mitochondria, or pyriproxyfen, which is a juvenile hormone analogue, 'non-standard' endpoints such as delayed mortality and insect fertility and fecundity need to be measured to assess biological durability (bioefficacy, measured through direct impact on mosquitoes).

Several of these new types of ITN are being evaluated in large-scale randomized control trials (RCTs) and pilot deployment schemes. These trials are expected to demonstrate the biological durability, attrition, and fabric integrity of these new net types when under long-term household use. Measuring the biological durability of the ITNs involves assessing the insecticidal activity of a sub-sample of randomly selected nets withdrawn from the field. There is an urgent need for methods to reliably measure the bioefficacy of these nets, to collect baseline data, and to subsequently measure the durability of biological efficacy of nets collected from the field after fixed periods of use. This has resulted in methods for measuring net bioefficacy and biological durability being developed and utilized by multiple programme teams, which makes comparing the results of these studies complex. A better approach would be for programme teams to adopt a single, standardized method validated using a multi-site approach.

In this publication, we demonstrate the process used to collate and interrogate several different methods to produce a singular 'consensus standard operating procedure (SOP)', for evaluating the biological efficacy of new net types, suitable for durability monitoring. Our objective was to create procedures that build on the experience from studies already underway. We also considered the feasibility of conducting these methods in as many sites as possible, accounting for factors such as throughput of mosquito colonies and space, which can preclude the use of certain methods and inform choices about sample sizes and replicate numbers.

This project forms part of a package of work to improve entomological methods in vector control and is supported by Innovation to Impact (I2I) at the Liverpool School of Tropical Medicine (LSTM). Three new types [7] of ITN are used as case studies: pyrethroid + piperonyl butoxide (PBO), pyrethroid + pyriproxyfen (PPF), and pyrethroid + chlorfenapyr (CFP). The final consensus SOPs for measuring the biological durability of these net types are included in Additional Files 2–4 (Supplementary materials).

2. Materials and Methods

For each net type, a collaborative process of method development and iterative drafting was conducted to produce a consensus SOP (Figure 1). Initially, a group of stakeholders was formed. Inclusion in these groups was based on having (1) a research interest in the development or deployment of new net types, (2) experience in the development or testing of new net types, or (3) an involvement in ongoing trials or deployment schemes of new net types. Available methods for measuring the biological durability of each net type were then identified through consultations with stakeholder groups and literature searches. This was not a systematic process, and for each net type, several historical procedures exist which were not considered here. Rather, the focus was to identify SOPs currently being developed or utilized which evaluated the biological durability of new net types and to use them to align the methods on points of difference. For each net type, the experimental parameters of the method were established (i.e., exposure method, controls used, population, replicates, endpoints). Values for each parameter were extracted from all accessible methods and compared before a 'consensus value' was suggested for each experimental element. Other methodological questions were identified for discussion. At this stage, the method development document was shared with the stakeholder group for comment, and further discussed on a group call. The feedback on the method development was then used to prepare a draft consensus SOP. The draft was distributed with the group for a second round of comments and discussion. Following the incorporation of this feedback, a final consensus SOP was produced and submitted to the group for approval.

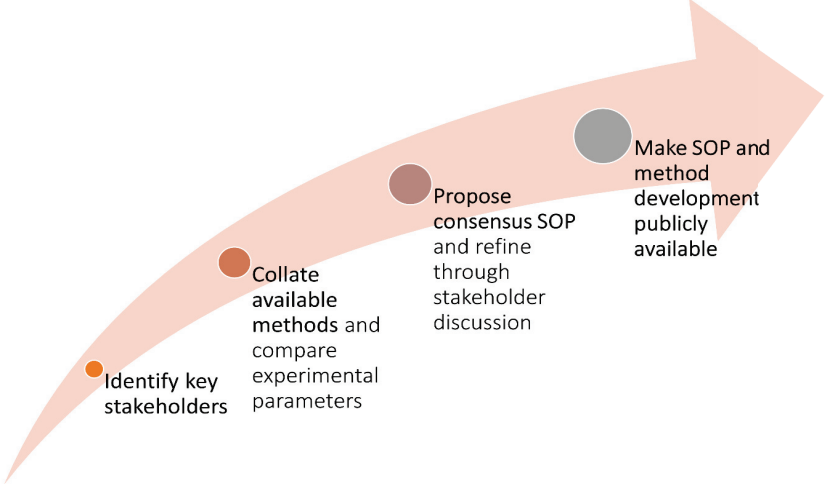

Figure 1. Infographic showing the process of method development used for producing consensus SOPs for biological durability monitoring of new net types.

3. Case Study 1: ITNs Containing Pyrethroid plus Piperonyl Butoxide (Pyrethroid + PBO Nets)

Currently, six pyrethroid + PBO nets are prequalified by the WHO (DuraNet Plus, VEERALIN, PermaNet 3.0, Tsara Boost, Tsara Plus, Olyset Plus) [3]. These vary in several

specifications (Additional File 1: Table S1) such as pyrethroid AI, PBO concentration, and location of PBO on the net (roof only or on all panels). A conventional cone test, followed by a tunnel test for those nets which fail to reach cone bioassay thresholds [8], is suitable for exposing mosquitoes to pyrethroid + PBO nets and monitoring mortality. Certain methodological parameters of the WHO cone test, such as replicate number and control nets, vary depending on if the assay is being used for WHOPES (the precursor to WHO prequalification) phase I, II, or III testing. The WHO guidance states "candidate LNs (nets) treated with insecticides with effects on mosquitoes that differ from those of pyrethroids may require proof of principle and new assays" [8]; however, guidance or thresholds on how to interpret PBO-synergism for biological durability monitoring is not available.

Nine methodologies that measure pyrethroid + PBO net biological durability were identified through searching the literature and contacting key stakeholders (Table 1). Of these, methods were accessible for six of them (published or provided on request). Of the remaining three, one study had not yet finalized its methods (ID = 7), one confirmed it was not conducting biological durability monitoring (ID = 8), and one did not have biological durability monitoring listed as an intervention endpoint on its clinical trial registry; the authors were contacted to confirm this, but they did not respond (ID = 9). Values for each methodological parameter were extracted from the accessible SOPs and a 'consensus' value suggested for each parameter (Table 2). It was established that one method (ID = 2) was an updated version of another (ID = 1), so study #2 was later excluded.

Table 1. List of identified methods/trials measuring pyrethroid + PBO net biological durability.

ID	Contact	Biological Durability Monitoring	Method Availability
#1 PMI VectorLink SOP for NNP	Stephen Poyer, PSI	Yes	Provided
#2 NNP Burkina Faso DM protocol	Stephen Poyer, PSI	Yes	Provided
#3 LLINEUP trial Uganda	Amy Lynd, LSTM	Yes	Provided
#4 LLINEUP trial LSTM	Frank Mechan, LSTM	Yes	Provided
#5 Nigeria trial (Awolola et al., 2014)	Samson Awolola, NIMR	Yes	Published
#6 Kenya SMART Trial NCT04182126	Guiyun Yan, UC Irvine	Yes	Provided
#7 ISRCTN99611164	David Weetman, LSTM	Yes	Method not set
#8 JPRN-UMIN000019971	Noboru Minakawa, Nagasaki University	No	-
#9 NCT03289663	Gillon Ilombe, University of Kinshasa	Unclear	-

Abbreviations: DM = Biological durability monitoring; LSTM = Liverpool School of Tropical Medicine; NIMR = Nigerian Institute of Medical Research; NNP = New Nets Project; PMI = President's Malaria Initiative; PSI = Population Services International; SOP = Standard operating procedure.

3.1. Other Methodological Considerations Identified

- Date, temperature, relative humidity, test species/strain (including resistance profiles), and mosquito age (days) should always be recorded.
- Time of testing and light–dark cycle of test mosquitoes should be recorded.
- Nets and mosquitoes should be acclimatized to the temperature and humidity of the testing room for a minimum of 1 h before testing. This is critical if nets have been stored in a refrigerator or cold room.
- For mosquitoes collected as larvae from the field, details on the collection procedure, such as the number and distribution of collection sites, and mosquito-rearing conditions, should be recorded.
- Some pyrethroid + PBO nets have different pyrethroid concentrations on the sides and the roof and this should be considered in the data recording and interpretation. Therefore, it is important that net pieces are well labelled to establish if the sample is from the roof or sides, and data should be recorded per net piece. Though analysis should be pooled for each net for interpretation, having the data disaggregated in this way will allow for further interrogation of the data if required.

Table 2. Methodological parameters extracted from pyrethroid + PBO net biological durability monitoring methods. Methods were compared and a consensus value was proposed for each parameter for discussion by the stakeholder group. Justification for this choice regarding each parameter is listed. Superscript numbers = Study ID.

	PMI VectorLink SOP [1]	LLINEUP SOPs [3]	Mechan PhD Project [4]	Nigeria Trial [5]	Kenya SMART Trial [6]	Proposed for Consensus SOP	Justification
Author	PMI VectorLink	Lynd (LSTM)	Mechan (LSTM)	Awolala (Nigeria medical institute)	Yan (University of California)	Lees and Lissenden (LSTM)	-
Method of exposure (primary test)	Cone (3 min)	Cone (3 min)	Cone (3 min)	Cone (3 min)	Cone (3–5 min)	Cone (3 min)	This is the standard exposure time used in WHO cone bioassays [6].
Controls	Untreated net. New pyrethroid-only net. New pyrethroid + PBO net.	Untreated net control.	-	-	Untreated net control.	Negative control: Untreated control net. Positive control 1: New pyrethroid + PBO net of the same brand. Positive control 2: Pyrethroid-only net of the same pyrethroid (as similar as possible).	Untreated net controls for handling procedure and checks for contamination. New pyrethroid + PBO net provides 'baseline' mortality and allows us to monitor the suitability of test mosquito strains. New pyrethroid-only net controls for the mortality conferred by the pyrethroid product.
Age of mosquito	-	3–5 days	3–5 days	2–3 days	2–5 days	2–5 days	Age range recommended for bioefficacy testing [6]. It encompasses the age ranges previously tested and is logistically feasible.
Mosquitoes per rep	5	5	5	5	10	5	This is the standard number used in WHO cone bioassays [6]

Table 2. *Cont.*

	PMI VectorLink SOP [1]	LLINEUP SOPs [3]	Mechan PhD Project [4]	Nigeria Trial [5]	Kenya SMART Trial [6]	Proposed for Consensus SOP	Justification
Samples per net	PBO all over: 4 pieces (1 roof); PBO roof only: 6 pieces (3 roof).	2 pieces from the top of the net (though 3 pieces were cut from net).	2 pieces from the top of the net (25×25 cm^2).	5 pieces (1 top, 4 sides).	5 pieces (1 top, 4 sides); 30×30 cm^2.	4 pieces (2 from net roof, 2 from net sides).	This aligns with the other new net type SOPs, and with the standard WHO biological durability testing where (post-baseline) 4 pieces of net are tested [6]. The decision to take equal pieces from the roof is due to greater mosquito activity observed here [9–11] and because some nets only have PBO on the roof. During their manufacture, roof panels can come from different net runs than side panels [12].
Replicate tests per piece of net	2 cones per net piece; PBO all over: $n = 40$; PBO roof only: $n = 60$.	25 per piece ($n = 50$).	3 cones simultaneously on each piece of net (6 cones total, $n = 30$).	1 cone per piece (25 mosquitoes).	2 cones per rep ($n = 100$ mosquitoes).	2 replicates per piece (8 cones per net).	Likely to be a feasible number for testing. Numbers will be finalized during multicenter validation of the SOP.
Replicate nets per treatment	-	-	-	+30 (35 houses selected).	18 nets	A minimum of 30 nets of each treatment at each time point.	WHO guidelines [6] recommend a minimum of 30 nets (at time points 0–24 months), and a minimum of 50 nets at 36 months testing.
Species/strain	A pyrethroid-susceptible and a pyrethroid-resistant strain.	-	A pyrethroid-susceptible (*An. gambiae* Kisumu) and a pyrethroid-resistant strain (*An. gambiae* Busia).	A pyrethroid-susceptible strain (*An. gambiae* Kisumu).	A pyrethroid-susceptible strain (*An. gambiae* Kisumu).	Lab-reared pyrethroid-susceptible strain. Lab-reared pyrethroid-resistant strain. Lab strains characterized before and after the bioassays for each time point, as per strain characterization guidelines (Lees et al. in prep).	The susceptible strain is used to monitor the biological durability of the pyrethroid over time. The pyrethroid-resistant strain is used to monitor the impact of PBO over time.

Table 2. *Cont.*

	PMI VectorLink SOP [1]	LLINEUP SOPs [3]	Mechan PhD Project [4]	Nigeria Trial [5]	Kenya SMART Trial [6]	Proposed for Consensus SOP	Justification
Storage of netting pieces (prior to testing)	-	Room temperature	Refrigerator-stored (5 °C)	-	In foil (4 °C)	Refrigerated or in a cool dry place, at <5 °C or as per manufacturer's instructions.	-
Entomological endpoints measured	Knock down (KD): 60 min; Mortality: 24 h.	KD: 60; Mortality: 24 h + alive with 2 or less legs, and the number alive and flying well with 3 or more legs.	KD: 60 min. Mortality: 24 h.	-	KD: 10, 20, 30, 40, 50, and 60 min. Mortality: 24 h.	KD: 1 h. Mortality: 24 h.	These endpoints are sufficient to capture the efficacy of a pyrethroid + PBO net.

3.2. Changes Made to the Proposed Pyrethroid + PBO Methods following Stakeholder Discussions

1. It was decided that it was clearer to structure the SOP based on net panel type (i.e., a pyrethroid-only net panel), rather than describe testing based on nets with 'PBO all over' vs. 'PBO mosaic net' (PBO on the roof only). This structuring should allow adaptation to ITNs that may be developed in the future with different net panel configurations.
2. Number of pieces sampled from each net: WHO biological durability monitoring [6] for pyrethroid-only nets recommended sampling one piece from the net roof and three–four pieces from the sides (four–five total). Our original proposal for pyrethroid + PBO nets was to sample three pieces from the roof and three from the sides (six total). The decision to test more roof samples was based on research which has shown greater mosquito activity on the net roof [9–11], the acknowledgement that some pyrethroid + PBO nets have different physio-chemical properties on the net roof, and that, during their manufacture, roof panels come from different net runs than side panels [12]. However, weighing up the benefits of a more precise measurement of intra-net heterogeneity by using six replicates per net against the challenge of evaluating large cohorts of ITNs with high numbers of mosquitoes per net, it was decided that the key measurement was the estimated bioefficacy of a cohort of ITNs. Therefore, it is important to be able to evaluate as many ITNs as possible (as nets have a high degree of heterogeneity due to different variability in use and care) while balancing this against the requirement for mosquitoes. Four samples per net (two from the roof, two from the sides) will allow the maximal numbers of samples to be tested without putting undue strain on testing facilities.
3. Replicates: The original proposal was four replicates per net sample based on the WHOPES recommendations for pyrethroid-only nets [6]. However, this made the required mosquito numbers unfeasible. The consensus was that two replicates per net sample was sufficient. If mosquito numbers are abundant, testing should prioritize testing more nets (if available), as this will provide more precision. If additional nets are not available, surplus mosquitoes could be used to conduct more test replicates. After the consensus SOP was developed, a pre-print was published [13], which contained additional methods for the planned evaluation of the biological durability of PBO nets. The methods published in that report were compared to the draft consensus SOP and, methodologically, these were found to be largely the same, with some variability in sampling position and number of net samples/replicates.
4. Testing should primarily use the WHO cone method specified in the consensus SOP (Additional File 2). A tunnel test may be used as a second test when nets fail to meet WHO thresholds (<95% 60-min knockdown or <80% 24-h mortality in a susceptible strain [6]), although this is not preferred. Currently, there are no recommended thresholds for resistant mosquito strains.

Following feedback from stakeholders, a final consensus SOP was produced and approved by the group (Additional File 2: I2I-SOP-001: Methods for monitoring the biological durability of insecticide-treated nets containing a pyrethroid plus piperonyl butoxide (PBO)).

4. Case Study 2: ITNs Containing Pyrethroid plus Pyriproxyfen (Pyrethroid + PPF Nets)

Royal Guard, developed by Disease Control Technologies, is currently the only WHO prequalification listed pyrethroid + PPF net (Additional File 1: Table S2). The WHO cone test is a suitable method for exposing mosquitoes to pyrethroid + pyriproxyfen (PPF) nets for measuring the nets' biological durability, but different endpoints are needed for each active ingredient. Knockdown and mortality can be used to assess the bio-efficacy of the pyrethroid but the most suitable endpoints for PPF, a juvenile hormone analogue that affects fertility and fecundity in mosquitoes, need to be defined.

Seven documents detailing methods for evaluating pyrethroid + PPF nets were provided by stakeholders (Table 3). One of these (ID = 1) did not measure fertility endpoints. Of the remaining documents, four detailed methods for oviposition observations, and two detailed methods for ovary dissection.

Table 3. List of identified methods/trials measuring pyrethroid + PPF net biological durability.

ID	Contact	Biological Durability Monitoring	Method Availability
#1 CNRFP tunnel test AvecNet	Emile Tchicaya, CSRS	Yes	N/A
#2 LSTM Cone test AvecNet (Toé et al., 2019)	Hyacinth Toé, CNRFP	Yes	Provided
#3 Oviposition SOP, CREC, Benin	Corine Ngufor, LSHTM	Yes	Provided
#4 Dissection SOP, CREC, Benin	Thomas Syme, LSHTM	Yes	Provided
#5 Dissection SOP, KCMUCO, Tanzania	Jackline Martin, KCMUCo	Yes	Provided
#6 Royal Guard Trial [14]	Corine Ngufor, LSHTM	Yes	Provided
#7 WHO PPF DC bottle study	Vincent Corbel, IRD	Yes	Provided

Abbreviations: CNRFP = Centre National de Recherche et de Formation sur le Paludisme; CREC = Centre de Recherche Entomologique de Cotonou; DC = Diagnostic concentration; IRD = Institute of Research for Development; KCMUCo = Kilimanjaro Christian Medical University College; LSHTM = London School of Hygiene and Tropical Medicine; LSTM = Liverpool School of Tropical Medicine; PPF = Pyriproxyfen; SOP = Standard operating procedure.

To reach a consensus SOP for both methods, methodological parameter values were extracted from available SOPs and a 'consensus' value was proposed for each one (Oviposition: Table 4; Dissections: Table 5). Methods for both oviposition and dissection are included, as discussions showed differences in preference between labs for one or the other method (Figure 2).

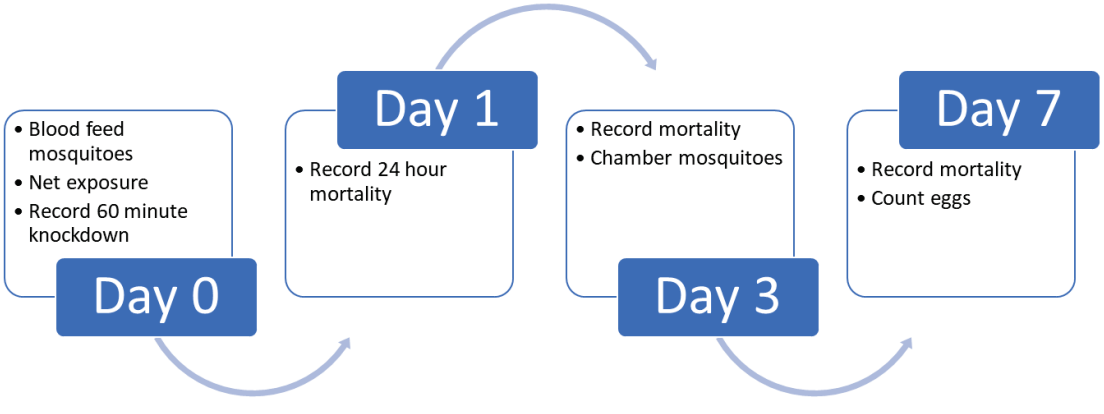

Figure 2. Infographic showing the methodological process for measuring sterility via scoring oviposition using chambering, following exposure to pyrethroid + PPF nets.

Table 4. Methodological parameters extracted from pyrethroid + PPF net biological durability monitoring methods, which scored mosquito oviposition. Methods were compared and a consensus value was proposed for each parameter. Justification for this choice regarding each parameter is listed. Superscript numbers = Study ID.

	Toé et al., 2019, Malaria Journal [1,2]		CREC, Benin SOP/BL/131/03-S [3]	Ngufor et al., 2020 Scientific Reports [6]		WHO SOP [7]	Proposed for Consensus SOP	Justification
Author(s)	Toé, Tchicaya, Ranson, Morgan, and Grisales		Gregbo, Fagbohou, and Ngufor	Ngufor		Corbel (based on LITE SOP)	Lees and Lissenden	-
Method of exposure	Cone Test	Tunnel Test (nets that did not reach target in cone test).	SOP-only covers post-exposure.	Cone Test	Tunnel Test	TGAI on bottles	Cone Test	The cone test has been used in several studies to evaluate PPF nets and seems to be a suitable method of exposure.
Exposure time	3 min	15 h, 18:00–09:00 h.	-	3 min	Overnight	1 h	3 min	This is the standard exposure time used in WHO cone bioassays [6]. Preliminary validation testing will be conducted to look at effect of exposure time.
Controls	Untreated net (4 reps per day, n = 20 mosquitoes). PPF-only net (4 reps per day, n = 20 mosquitoes).	Untreated netting.	-	Royal Sentry (alpha-cypermethrin net). Untreated control net.	Royal Sentry (alpha-cypermethrin net). Untreated control net.	Does not state treatment of control bottles.	Negative control: Untreated control net. Positive control: New pyrethroid + PPF net of the same brand.	Untreated net controls for handling procedure and checks for contamination, and provides denominator for measuring oviposition inhibition. New pyrethroid + PPF net provides 'baseline' and allows us to monitor the suitability of test mosquito strains.

Table 4. *Cont.*

	Toé et al., 2019, Malaria Journal [1,2]	CREC, Benin SOP/BL/131/03-S [3]	Ngufor et al., 2020 Scientific Reports [6]		WHO SOP [7]	Proposed for Consensus SOP	Justification
Species/strain	Kisumu (pyrethroid-susceptible) in CNRFP, Kisumu and Tiassalé 13 (pyrethroid-resistant) in LSTM. Sterilizing effect only tested in LSTM on Tiassalé 13 that survived the Cone Test.	Kisumu	-	Kisumu and pyrethroid-resistant *An. gambiae* Cove strain.	Kisumu and pyrethroid-resistant *An. gambiae* Cove strain.	Susceptible strains of each species.	Lab-reared pyrethroid-susceptible strain Lab-reared pyrethroid-resistant strain Lab strains characterized before and after the bioassays for each time point as per strain characterization guidelines (Lees et al. In prep).	Lab-reared strains increase the likelihood of forced oviposition, yielding high rates. Pyrethroid-susceptible strain to monitor pyrethroid durability. Pyrethroid-resistant strain to monitor durability of PPF.
Age of mosquitoes	3–5 days	5–8 days	-	2–5 days old	5–8 days	5–7 days old, fed and inseminated.	3–5 days	This age range falls within the range of standard cone test (2–5 days, [6]) but allows an extra day for mating to increase likelihood of insemination. Effect of age for PPF is unknown and could be validated, but should be held constant until it is.
Mosquitoes per replicate	5	100	-	5	~80	25/bottle, 2 bottles/concentration, equal numbers of controls.	5 per cone.	This is the standard number used in WHO cone bioassays [6].

Table 4. *Cont.*

	Toé et al., 2019, Malaria Journal [1,2]		CREC, Benin SOP/BL/131/03-S [3]	Ngufor et al., 2020 Scientific Reports [6]		WHO SOP [7]	Proposed for Consensus SOP	Justification
Samples per net	3 panels per net, one from each side at CNRFP, and 4 further panels for LSTM. 4 tests per panel at CNFRP, 3 further panels in LSTM.	'Nets that did not reach the target'.	-	1	1	-	4 pieces from each net. Two from the roof, two from the sides.	This aligns with the other next-gen net SOPs, and with the standard WHO durability testing where (post-baseline) 4 pieces of net are tested [6]. The decision to take equal pieces from the roof is due to greater mosquito activity observed here [9]. During their manufacture, roof panels can come from different net runs than side panels [12]
Replicate tests per piece of net	3	?	-	1	1	N/A	2 replicates per piece (8 cones per net).	Consensus was that this was a feasible number for testing. Numbers will be confirmed during multi-center validation.
Replicate nets per treatment	24 of each type, or as many as available (high attrition), per timepoint.	'Nets that did not reach the target'.	-	4 (2 control)	3	N/A	A minimum of 30 nets for each treatment at each time point.	WHO guidelines [6] recommend a minimum of 30 nets (at time points 0–24 months), and a minimum of 50 nets at 36 months testing.
Blood feeding timing	24 h post-exposure (LSTM: 30 min blood meal using Hemotek membrane feeding system).	-	Before exposure.	Before exposure (separate group b/d after exposure failed to feed and too few survived).	Unfed females used in test. Only blood-fed during tunnel were measured after for sterilizing effects.	Fed in the hour before exposure.	3–9 h before net exposure Blood fed using method of feeding standard for the test population (e.g., Hemotek membrane feeding system, arm feed, animal fed to repletion).	There is little data available and some contradiction on the impact of time of blood feeding, and this could be validated. Consensus was that this was a suitable and logistically possible method.

Table 4. *Cont.*

	Toé et al., 2019, Malaria Journal [1,2]		CREC, Benin SOP/BL/131/03-S [3]	Ngufor et al., 2020 Scientific Reports [6]		WHO SOP [7]	Proposed for Consensus SOP	Justification
Timing of chambering	24 h post-exposure (LSTM) 72-h post-bloodmeal, 96-h post-exposure	Sterilizing effect not measured.	-	-	-	72 h post-exposure (73 h post b/m).	72 h post-exposure (Day 3).	This allows 3 days for bloodmeal development and egg maturation.
Method of chambering	30-mL cell culture tubes, moist cotton wool, and filter paper, individuals. Chambered for 3 days.	-	Cup, 50 mL water, 10% glucose cotton wool, individuals.	Individuals	-	100-mL plastic cups, 30 mL water, 10% glucose, individuals.	The chambering equipment used (i.e., culture tubes or plastic cups) is not critical and should reflect what method each lab has capacity to conduct. The same setup should then be used for all treatments and replicates. When oviposition in the untreated control is <20%, test results should be discarded and repeated.	20% oviposition threshold in the untreated control is based on power calculations performed by Joe Wagman (PATH).
Entomological endpoints measured	KD: 60 min; Mortality: 24 h; Number blood-fed; Eggs laid per female; Number 2nd instar larvae per female; Oviposition rate, fecundity, hatch rate, and fertility.	Blood-fed and dead after test.	Daily mortality to day 8. Count eggs and larvae on day 4 and day 8.	KD: 60 min. Mortality: 24-h mortality, individual oviposition: % reduction in oviposition rate, % reduction in fecundity, % reduction in offspring.	# alive/dead and # fed/unfed in each section, 24-h mortality, individual oviposition: % reduction in oviposition rate, % reduction in fecundity, % reduction in offspring.	KD: 60 min. Daily mortality (pre- and post-chambering until Day 8). Presence of eggs on day 8 post-exposure. Oviposition rate. Oviposition inhibition.	Primary endpoint: oviposition inhibition (calculated compared to untreated control. Additional measures: KD: 60 min, 24-h mortality, 72-h mortality (when chambering). Oviposition (egg laying) counted on Day 7 post-exposure only (4 days post-chambering).	A preliminary validation test will be conducted to establish if other endpoints should be included, e.g., median number of eggs laid.

Table 4. *Cont.*

	Toé et al., 2019, Malaria Journal [1,2]		CREC, Benin SOP/BL/131/03-S [3]	Ngufor et al., 2020 Scientific Reports [6]		WHO SOP [7]	Proposed for Consensus SOP	Justification
Length of bioassay	-	15 h	8 days post-exposure.	-	-	8 days post-exposure.	8 days (Day 0 = day of exposure).	-
Notes on the protocol	High-performance liquid chromatography (HPLC) conducted on net samples—3 samples from each of 4 panels. Sterilizing effect measured in rounds 1–5 (1–24 m).	Untreated control run for each round.	No food provided to eggs/larvae. Water with eggs transferred to larvae cup on day 4.	-	-	Test rejected if control mortality is 20% or more, or oviposition in controls is <30%.		-
Storage of netting pieces (prior to testing)	-	-	-	-	-	-	Refrigerated or in a cool dry place, but at <5 °C or as per manufacturer's instructions.	-

Table 5. Methodological parameters extracted from pyrethroid + PPF net biological durability monitoring methods, which scored ovary development following dissection. Methods were compared and a consensus value was proposed for each parameter. Justification for this choice regarding each parameter is listed. Superscript numbers = Study ID.

	CREC, Benin SOP BL/159/01-S v01 [4]	KCMUCO, Tanzania SOP 008v02 [5]	Proposed for Consensus SOP	Justification
Author	Syme	Martin, Matowo, and Furnival-Adams	Lees and Lissenden	-
Method of exposure	Not included in SOP	Cone test	Cone test	The cone test has been used in several studies to evaluate PPF nets and seems to be a suitable method of exposure.
Exposure time	Not included in SOP	3 min	3 min	This is the standard exposure time used in WHO cone bioassays [6]. Preliminary validation testing will be conducted to look at effect of exposure time.

Table 5. *Cont.*

	CREC, Benin SOP BL/159/01-S v01 [4]	KCMUCO, Tanzania SOP 008v02 [5]	Proposed for Consensus SOP	Justification
Age of mosquitoes	Unknown	2–5 days old	3–5 days	This age range falls within the range of standard cone test (2–5 days, [6]) but allows an extra day for mating to increase likelihood of insemination. Effect of age for PPF is unknown and could be validated, but should be held constant until it is.
Blood feeding timing	'Blood-fed at the time of collection/testing'.	Females 'freshly blood fed' for exposure.	3–9 h before net exposure. Blood fed using method of feeding standard for the test population (e.g., Hemotek membrane feeding system, arm feed, animal feed).	There is little data available and some contradiction on the impact of time of blood feeding, and this could be validated. Consensus was that this was a suitable, and logistically possible, method.
Mosquitoes per replicate	N/A	5	5 per cone	This is the standard number used in WHO cone bioassays [6].
Replicates per piece of net	N/A	20–25 replicates (n = 100–150); 4 per piece; 30 nets per treatment.	2 replicates per piece (8 cones per net)	Consensus was that this was a feasible number for testing. Numbers will be confirmed during multi-center validation.
Replicate nets per treatment	N/A		A minimum of 30 nets of each treatment at each time point.	WHO guidelines [6] recommend a minimum of 30 nets (at time points 0–24 months), and a minimum of 50 nets at 36 months testing.
Species/strain	*Anopheles* mosquitoes (generic SOP for dissection).	*An. gambiae* s.s. Muleba kis (kdr east and mixed-function oxidize resistance), or wild blood-fed resistance mosquitoes of unknown age with species id at time of dissection.	Lab-reared pyrethroid-susceptible strain. Lab-reared pyrethroid-resistant strain. Lab strains characterized before and after the bioassays for each time point as per strain characterization guidelines (Lees et al. in prep).	Pyrethroid-susceptible strain to monitor pyrethroid durability. Pyrethroid-resistant strain to monitor durability of PPF.

Table 5. *Cont.*

	CREC, Benin SOP BL/159/01-S v01 [4]	KCMUCO, Tanzania SOP 008v02 [5]	Proposed for Consensus SOP	Justification
Time of dissection	72 h post-exposure	72 h post-exposure	72 h post-exposure	This allows 3 days for bloodmeal digestion and egg maturation.
Blinded samples	No	Yes	Yes	Controls for scorer subjectivity.
Number of scorers	2, in case of discrepancy calculate the average (only for egg count).	2, using slide or photograph if slide cannot be counted on the same day. 3 scorers in case of discrepancy.	2, using slide or photograph if slide cannot be counted on the same day. 3rd scorer in cases of discrepancy.	Controls for scorer subjectivity.
Microscope details	Can use dissecting microscope, better a compound microscope at 4× or 10×.	0.7× magnification, stereomicroscope.	Microscope details not critical. However, we recommend using a magnification of ×4 or ×10 for dissections and ×40 for observation of eggs.	-
Entomological endpoints measured	Live/dead and gravid/semi-gravid at time of collection, egg development stage, and fertility status of each mosquito, total number of eggs present in ovary (1/2 per female?).	KD: 60 min. Mortality: 24 h. Mortality: 48 h. Mortality: 72 h. % of dissected females with under-developed ovaries 72 h post-feeding. Proportion of dissected females with deformed eggs. Average number of eggs in the ovaries 72 h post-feeding.	Primary endpoint: Fertility inhibition (fertility rate/fertility rate in the negative control). Additional measures: KD: 60 min. 24-h mortality. Egg development stage. Fertility rate (proportion with developed ovaries/total).	A preliminary validation test will be conducted to establish if other endpoints should be included, e.g., number of eggs in each dissected ovary.
Definition of Fertility	Christophers' scale to score development stage of eggs (I–V); female is fertile if eggs are V and sterile if eggs are I–IV.	Christophers' stages to score development stage of eggs (I–V); female is fertile if eggs are V and sterile if eggs are I–IV. Inconclusive if both are present.	Score development stage of eggs (1–5) [15]. Female is classed as fertile if all eggs are 5 and sterile if eggs are 1–4. If both classes 4 and 5 are present, the results are inconclusive.	This is a well-established method for scoring fertility

Table 5. *Cont.*

	CREC, Benin SOP BL/159/01-S v01 [4]	KCMUCO, Tanzania SOP 008v02 [5]	Proposed for Consensus SOP	Justification
Controls	-	Untreated net. Standard LN: Interceptor.	Negative control: Untreated control net. Positive control 1: New pyrethroid + PPF net of the same brand.	Untreated net controls for handling procedure and checks for contamination, and provides denominator for measuring oviposition inhibition. New pyrethroid + PPF net provides 'baseline' and allows us to monitor the suitability of test mosquito strains.
Notes on the protocol	Dissect all mosquitoes left alive at 72 h post-collection, but if there are not adequate numbers, also dissect dead mosquitoes at this time. Photographs taken of eggs.	Method from Detinova et al. 1962. Photographs taken of eggs.	If the testing site has the capacity to photograph dissected ovaries, then this should be conducted. Photographs can then be used in future training, and machine learning activities.	

Changes Made to the Proposed Methods following Stakeholder Discussions

1. The option to score oviposition and then dissect those that did not lay was discounted. This would have meant dissections were being conducted on non-standardized days, making results incomparable to data collected using the standard dissection method, and likely resulting in a small sample size for that subset. For similar reasons, those which died before oviposition counts should not be dissected and scored.
2. As we do not expect the pyrethroid to impact fertility, and we are using a pyrethroid-resistant strain, the untreated net is a useful negative control, and oviposition inhibition can be compared to this. Therefore, the decision was made not to include a pyrethroid-only net.
3. Questions remain regarding the 'net effectiveness threshold' for sterility endpoints. For pyrethroid only nets, a net is considered effective if KD_{60} is >95% or 24-h mortality is >80% [6]. We do not yet know what an operationally meaningful level of sterility is, i.e., what level of sterility in a cone test means the net is controlling mosquitoes in the field. Hence, it is not yet possible to set a threshold for biological durability monitoring, and the best approach is to simply monitor for a reduction in sterilizing effect over time. However, this question is critical and should be considered as data is generated.
4. When analyzing the results, the untreated net and the test net should be paired, i.e., a single control for the day acts as the benchmark for all tests on that day, and inhibition is calculated against that day's control. Inhibition can be calculated by odds ratio using regressions.
5. Following the development of the consensus SOP, a pre-print was published, which contained additional methods planned for evaluating biological durability of PPF nets [13]). These methods were compared to the drafted consensus SOP and found to be methodologically the same, apart from some variability in sampling position and number of net samples/replicates.

Following feedback from stakeholders, a final consensus SOP was prepared and approved by the group (Additional File 3: I2I-SOP-002: Methods for monitoring the biological durability of insecticide-treated nets containing a pyrethroid plus pyriproxyfen (PPF)).

5. Case Study 3: ITNs Containing Pyrethroid plus Chlorfenapyr (Pyrethroid + CFP Nets)

Interceptor G2 (IG2), developed by BASF, is currently the only WHO prequalification listed pyrethroid + CFP net (Additional File 1: Table S3). The cone test has been shown to be ineffective in reliably measuring the bioefficacy of the chlorfenapyr component of IG2 nets [16], and so an alternative bioassay is needed. There is a growing consensus around the WHO tunnel test as being the best method to assess IG2 bioefficacy. This should be run in parallel with a standard WHO cone test [6], which assesses the biological durability of the alpha-cypermethrin component of the net. The SOP discussed and included (Additional File 4) here is related to assessing the biological durability of the CFP component.

Eight documents, detailing methods used for evaluating pyrethroid + CFP nets, were provided by stakeholders (Table 6). Of these, three were generic SOPs for conducting the 'net in tube' cylinder assay (ID = 6) or tunnel test (ID = 7, 8), and did not contain specific experimental parameters for testing CFP nets, and, therefore, information was not extracted from them for comparison. Methodological parameters were extracted from the available SOPs, compared, and used to propose a 'consensus' value for each (Table 7).

Table 6. List of identified methods/trials measuring pyrethroid + CFP net biological durability.

ID	Contact	Biological Durability Monitoring	Method Availability
NNP Burkina Faso DM (ID = 1)	Richard Oxborough, PMI	Yes	Provided
Tanzania cRCT (Martin et al., 2021) (ID = 2)	Jackline Martin, KCMUCo	Yes	Published pre-print
Net in tube CFP, LSTM (ID = 3)	Katherine Gleave, LSTM	Yes	Provided
PMI CFP Tunnel SOP (ID = 4)	Richard Oxborough, PMI	Yes	Provided
Residual efficacy of Interceptor G2 (ID = 5)	Seth Irish, CDC, and Richard Oxborough, PMI	Yes	Provided
PAMVERC SOP for cylinder assay (ID = 6)	Leslie Choi, LSTM	Yes	N/A, generic SOP
IT LN SOP 002 V04—Tunnel Tests (ID = 7)	Sarah Moore, IHI	Yes	N/A, generic SOP
CREC SOP.BL.112.05.S—Tunnel tests (ID = 8)	Corine Ngufor, LSHTM	Yes	N/A, generic SOP

Abbreviations: CFP = Chlorfenapyr; cRCT = Cluster Randomized Control Trial; CREC = Centre de Recherche Entomologique de Cotonou; IHI = Ifakara Health Institute; KCMUCo = Kilimanjaro Christian Medical University College; LSHTM = London School of Hygiene and Tropical Medicine; LSTM = Liverpool School of Tropical Medicine; NNP = New Nets Project; PAMCERC = Pan-African Malaria Vector Research Consortium; PMI = Presidents Malaria Initiative; SOP = Standard operating procedure.

Table 7. Methodological parameters extracted from pyrethroid + chlorfenapyr net biological durability-monitoring methods. Methods were compared and a consensus value was proposed for each parameter. Justifications for this choice, regarding each parameter, are listed. Abbreviations: IG1 = Interceptor Net, Alpha-cypermethrin net; IG2 = Interceptor G2, Chlorfenapyr + Alpha-cypermethrin net. Superscript numbers = Study ID.

	NNP Burkina Faso DM [1]	Tanzania cRCT [2]			Net in Tube, LSTM [3]	PMI SOP [4]	Irish and Oxborough SOP [5]	Proposed for Consensus SOP	Justification
Author(s)	NNP	JL Martin et al.			Irish, Oxborough & Gleave	PMI	Irish and Oxborough	Lissenden	
Method of exposure (primary test)	Cone test	Cone test	Tunnel test	Cylinder test	Cylinder test	Tunnel Test	Tunnel Test	Tunnel Test	The tunnel test has been used in several studies to evaluate CFP nets and seems to be a suitable method of exposure.
Exposure time	-	3 min	12–15 h	3, 15, 30, 60 min, 'as necessary'	30 min		12–15 h	12–15 h	This is the standard exposure time used in WHO tunnel tests [6].
Controls	No exposure control	Untreated net IG1 collected at same time point.	Untreated net IG1 collected at same time point.	-	Untreated net Alphacypermethrin net (100 mg/m^2).	Negative control New IG1 New IG2	Untreated net. New IG1. New IG2 (used up to 10 times).	Untreated net (Used up to 10 times). Untreated Control thresholds: blood-feeding must be >50%. Mortality must be <10% after 24 h and < 20% at 72 h. New IG1 and IG2 should be used to characterize strain prior to testing.	Untreated net controls for handling procedure and checks for contamination and provides denominator for measuring oviposition inhibition. New IG1 + IG2 nets provides 'baseline' and allows us to monitor the suitability of test mosquito strains.

Table 7. *Cont.*

	NNP Burkina Faso DM [1]	Tanzania cRCT [2]		Net in Tube, LSTM [3]	PMI SOP [4]	Irish and Oxborough SOP [5]	Proposed for Consensus SOP	Justification
Species/ strain	Pyrethroid-susceptible strain. Pyrethroid-resistant strain.	A pyrethroid-susceptible strain (Kisumu).	A pyrethroid-susceptible strain (Kisumu—failed cone nets only). Pyrethroid-resistant strain (Muleba-kis), regularly selected and profiled.	-	Pyrethroid-resistant strain (<70% mortality).	Pyrethroid-susceptible (Kisumu) strain Pyrethroid-resistant (VKPER) strain	Profiled pyrethroid-resistant strain (<70% mortality to new IG1).	Lab-reared pyrethroid-susceptible strain. Lab-reared pyrethroid-resistant strain. Lab strains characterized before and after the bioassays for each time point, as per strain characterization guidelines (Lees et al. In prep).	The susceptible strain is used to monitor the biological durability of the pyrethroid over time. The pyrethroid-resistant strain is used to monitor the impact of CFP over time.
Age of mosquito	2–5 days	2–5 days	-	-	3–5 days	-	5–8 days old	5–8 days	This is the standard age used in WHO tunnel tests [6].
Status of mosquito	Unfed	-	-	-	Non-blood-fed; Sugar-starved, 6 h.		Nulliparous. Sugar-starved, 6 h	Nulliparous. Non-blood-fed. Sugar-starved for a minimum of 6 h.	This is the standard mosquito status used in WHO tunnel tests [6]. Consensus agreed sugar-starving found increase mosquito responsiveness to bait.
Mosquitoes per replicate	5	5	50	10	20–25		100	50	Preliminary research has shown no difference between using 50 or 100 mosquitoes in tunnel tests with IG2 (Kamande, Personal communication).

Table 7. *Cont.*

	NNP Burkina Faso DM [1]	Tanzania cRCT [2]		Net in Tube, LSTM [3]	PMI SOP [4]	Irish and Oxborough SOP [5]	Proposed for Consensus SOP	Justification
Samples per net	2 (30 × 30 cm)	Baseline: 5 pieces (1 top, 4 sides). Post-baseline: 4 pieces (1 top, 3 sides).	1 piece (position 2), 25 × 25 cm, 9 × 1 cm holes.	-	4 tubes (4 net pieces).		4 (30 × 30 cm)	2 pieces (1 from roof, 1 from sides); 30 × 30 cm, 9 × 1 cm holes in net.	In the standard WHO tunnel test, one net piece is used [6]. The increase allows a 2nd piece from the roof to be tested. During their manufacture, roof panels can come from different net runs than side panels [12].
Replicate tests per piece of net	2	4 replicates	2 replicates	-	1 replicate per net.		?	1 replicate per net piece.	This is the standard used in WHO tunnel tests [6].
Replicate nets per treatment	30	30 nets (timepoint: 0–30 months), 50 nets (timepoint: 36 months).	30 nets (timepoint: 0–30 months), 50 nets (t36).	Sub-set of nets	2 per testing day (200–250 mosquitoes).			A minimum of 30 nets for each treatment at each time point.	WHO guidelines [6] recommend a minimum of 30 nets (at time points 0–24 months), and a minimum of 50 nets at 36 months testing.
Storage of netting pieces (prior to testing)	cool dry place at 4°	-	-	-				Refrigerated or in a cool dry place, at <5 °C or as per manufacturer's instructions.	-
Entomological endpoints measured	KD: 30 min. KD: 60 min. Mortality: 24 h.	KD: 60 min. Mortality: 24 h. Mortality: 48 h. Mortality: 72 h.	KD: 60 min. Mortality: 24 h. Mortality: 48 h. Mortality: 72 h. Blood feeding.	-	KD: 60 min. Mortality: 24 h. Mortality: 48 h. Mortality: 72 h.	Mortality: 24 h. Mortality: 72 h. Net penetration. Blood feeding. Blood feeding inhibition. Corrected mortality due to chlorfenapyr.	Collection compartment. Blood-feeding status. 'Immediate' mortality (07:00). 'Delayed' mortality 24 h, 48 h, 72 h.	Collection compartment. Blood-feeding status. Mortality on collection ('immediate'). 24 h, 48 h, 72 h mortality ('delayed').	These endpoints are sufficient to capture the efficacy of a pyrethroid + CFP net.

Table 7. *Cont.*

	NNP Burkina Faso DM [1]	Tanzania cRCT [2]		Net in Tube, LSTM [3]	PMI SOP [4]	Irish and Oxborough SOP [5]	Proposed for Consensus SOP	Justification
Other	Cone test is only looking at impact of alpha-cypermethrin.	18:00: introduced; 08:00: end.	-	Conducted in darkness during the 'night phase' of mosquitoes' circadian rhythm; 27 ± 2 °C and 75% ± 10% relative humidity. Acclimatized to holding tubes for 1 h.		18:00: introduced; 07:00: end. Conducted in darkness, 27 ± 2 °C and 75% ± 10% relative humidity. Mortality corrected for alpha mortality.	Conducted in darkness during the 'night phase' of the mosquitoes' circadian rhythm. Blood meal source preferably the same as what was used to feed the strain in colony, 27 ± 2 °C and 75% ± 10% relative humidity.	Higher mortalities have been observed when chlorfenapyr is used overnight [16], when, as a result of the *Anopheles* circadian rhythm, flight is increased, and, subsequently, cellular respiration and oxidative metabolism, which the chlorfenapyr targets ([17]), is at its peak.

Changes Made to the Proposed Pyrethroid + CFP Methods following Stakeholder Discussions

1. Where tunnel testing is not possible, it would be beneficial to have an additional method available. It was established that S. Moore will be validating the I-ACT method [18] for IG2 testing, and K. Gleave will be validating the 'Net in Tube' (cylinder) test. When complete, we will include these SOPs with the tunnel-test methodology on the I2I website (https://innovationtoimpact.org/workstreams/methods-validation/). Accessed on 20 December 2021.
2. Following a preliminary discussion with all stakeholders, a sub-group was formed with key individuals to start a draft proposal for the CFP methodology. In the initial meeting, representatives of BASF joined to share information on Interceptor G2. Following on from these discussions, a draft method development with methodological parameters for the tunnel test was shared with the sub-group, and this was refined before sharing with the full stakeholder group for approval.
3. From a biological durability perspective, it was decided that it was not necessary to have a comparison to a new Interceptor net (IG1) and a new Interceptor G2 net (IG2) at every time point. Thus, these were removed as daily controls. Instead, the resistant strain should be characterized against the Ais in parallel with each round of bioassays, as recommended in Lees et al. (in prep), to investigate the additional effect of chlorfenapyr, and to confirm pyrethroid resistance and chlorfenapyr susceptibility to check that they have not drifted in the test strain during the test period.
4. There is a lack of data on how mortality in tunnel tests changes with mosquito numbers (the standard is 100 mosquitoes in a tunnel). Reducing the sample to 50 mosquitoes per tunnel allows us to increase the sample pieces tested per net without increasing mosquito numbers. However, this also increases the risk of having to disregard testing results if high control mortality is observed—control mortality would still be based on 100 mosquitoes, but over two net replicates.
    a. Data comparing the use of 50 vs. 100 mosquitoes in tunnels with pyrethroid nets are available (Moore, Personal communication), and these data were considered to confirm the number of mosquitoes tested.
    b. Further to this, preliminary work to compare 50 vs. 100 mosquitoes in tunnels using Interceptor net and Interceptor G2 nets was conducted, and found no significant difference in these two numbers (Kamande, Personal communication).
5. The number of mosquitoes required must be balanced against the number of replicates, since maximizing the number of nets, to measure efficacy of the ITN population, is key. There was some disagreement over which was the best balance. It is likely that the capacity to test more mosquitoes per net will be related to mosquito availability in the testing sites. Therefore, it is suggested we validate with the lower number to make the SOP less onerous for testing sites. We are interested in measuring the biological durability of the ITN population—not individual nets, which could be highly variable. Currently, the WHO recommends 30 nets per time point, but increasing this will provide better data. Thirty nets should be seen as the minimum. Reducing the number of mosquitoes may allow increases in replication to be possible.
6. Control thresholds: blood-feeding must be >50% on the untreated control net. Mortality will be measured up to 72 h, due to the slow-acting nature of chlorfenapyr. Mortality in the untreated control must be <10% after 24 h and <20% at 72 h (both must be true for the test to be valid).

Following feedback from stakeholders, a final consensus SOP was produced and approved by the group (Additional File 4: I2I-SOP-003: Methods for monitoring the biological durability of insecticide-treated nets containing chlorfenapyr).

6. Discussion

Methodological consistency is crucially important when monitoring the durability of new net types, due to there not being validated methods to assess these tools. Even small

differences in testing methods may lead to additional sources of variation in endpoints, making results difficult to interpret between countries, studies, and test facilities. The use of standardized testing methods streamlines the process of product evaluation, leads to a more rapid generation of consistent performance data across studies, and subsequently speeds up product uptake. In vector control, methods for new tools with novel modes of action are often developed in one site or by one group in response to a specific product or research question. This can narrow the applicability of that method, make it challenging to adopt it at other sites, or it may not be applicable to all products within a particular product class.

Developing evaluation methods in a collaborative group ('consensus' SOPs) allows the process to benefit from the collective knowledge and experience of a diverse set of stakeholders, and maximizes the chances for a specific methodology that will be widely relevant. However, developing a consensus SOP is just one of the first steps in the method-validation pipeline. Defining and improving the robustness of a method can be viewed as an incremental process which follows a stepwise progression from singular SOPs to consensus SOPs, to consensus SOPs that are experimentally validated at one site, and finally, to consensus SOPs that are validated at multiple sites. In this publication, we have defined the desired endpoints, and designed and refined methodologies for evaluating the biological durability of three new net types. The next steps in this process will be to (1) quantify inherent errors in the methods, (2) evaluate the ability of the methods to accurately characterize the vector control product, and (3) validate these results in multiple facilities. The scope of this would include assessing the methods' ability to measure the biological durability of different products within the class of nets, and against different vector species. More information is gathered when a method is in operational use, which can help to improve or refine the method. At this stage, it is imperative to ascertain that the methods can be implemented and used successfully within research teams, and identify training needs, if required. This is to ensure that data collected using these methods are as transferable and comparable as possible.

The agreement on key entomological endpoints to be measured, followed by the use of standardized and validated methods to measure them, needs to be partnered with an acceptance of the need for flexibility in product evaluation. For instance, the SOPs developed here have been formulated based on nets that are currently in development/on the market and therefore may be unsuitable for new formulations or designs within the same product classes. However, it should be noted that this is the way that previous ITN guidelines were developed—in response to new technologies coming to market [6]. It is challenging to 'future-proof' methods from the outset, especially in a rapidly evolving landscape which must be sensitive to the pressures of evolving and emerging insecticide resistance. Therefore, the process cannot be averse to change or updates in the future, which would lead to stagnation in innovation and delayed decision making—such has been the situation with non-pyrethroid products being evaluated with tests designed for pyrethroids. Regular updates of guidance based on consensus among key stakeholders will harmonize data collection procedures and, ultimately, hasten progress towards the goal of bringing new vector control products to market more rapidly, using robust data-driven decision making.

To take this further, the dissemination of up-to-date methods is crucial to ensure relevant data are being collected whenever possible. This process, convened under the auspices of the Innovation to Impact programme, sought to align methodologies used by those conducting durability monitoring activities of new net types (so-called 'next-generation ITNs'). While this objective was largely achieved through the engagement and insight of those involved, it is important to recognize that even though this process involved the key stakeholders in designing and implementing durability monitoring, the current durability monitoring guidelines [6] for these products may differ or simply do not exist. There is a clear need for further engagement with normative (WHO and control

programmes) and implementation groups (Roll Back Malaria and others) to ensure up-to-date guidance for durability monitoring is available to all who may wish to access it.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects13010007/s1, Additional File 1: Developing consensus SOPs for evaluating new types of insecticide-treated nets—supplementary tables and figures; Additional File 2: I2I-SOP-001: Methods for monitoring the biological durability of insecticide-treated nets containing a pyrethroid plus piperonyl butoxide (PBO); Additional File 3: I2I-SOP-002: Methods for monitoring the biological durability of insecticide-treated nets containing a pyrethroid plus pyriproxyfen (PPF); Additional File 4: I2I-SOP-003: Methods for monitoring the biological durability of insecticide-treated nets containing a pyrethroid plus chlorfenapyr (CFP)s.

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Guidelines

Strain Characterisation for Measuring Bioefficacy of ITNs Treated with Two Active Ingredients (Dual-AI ITNs): Developing a Robust Protocol by Building Consensus

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Simple Summary: New types of bed nets are being developed which contain a pyrethroid plus a second chemical because of the development and increased frequency of mosquito mechanisms to avoid being killed by pyrethroids. When insecticide-treated bed nets are being trialed for efficacy or released onto the market it is essential to measure how effective the net is in killing mosquitoes, which includes testing how quickly insecticide is lost or degraded due to routine wear and tear. Pyrethroid-resistant mosquitoes are needed to test the effectiveness and insecticidal persistence of the second chemical, because they are not all killed by the pyrethroid, allowing the killing effect of the two chemicals to be evaluated independently. However, because resistance status varies between populations of mosquitoes that possess different resistance mechanisms, and because resistance intensity in a population can change over time, a method is needed to characterise the resistant

mosquitoes. A focus group of experts discussed how this should best be done, considering pros and cons of different approaches, and co-wrote a protocol, which will be valuable for malaria control programmes and stakeholders wanting to test the effective lifespan of a new bed net in terms of the active ingredient bioefficacy.

Abstract: Durability monitoring of insecticide-treated nets (ITNs) containing a pyrethroid in combination with a second active ingredient (AI) must be adapted so that the insecticidal bioefficacy of each AI can be monitored independently. An effective way to do this is to measure rapid knock down of a pyrethroid-susceptible strain of mosquitoes to assess the bioefficacy of the pyrethroid component and to use a pyrethroid-resistant strain to measure the bioefficacy of the second ingredient. To allow robust comparison of results across tests within and between test facilities, and over time, protocols for bioefficacy testing must include either characterisation of the resistant strain, standardisation of the mosquitoes used for bioassays, or a combination of the two. Through a series of virtual meetings, key stakeholders and practitioners explored different approaches to achieving these goals. Via an iterative process we decided on the preferred approach and produced a protocol consisting of characterising mosquitoes used for bioefficacy testing before and after a round of bioassays, for example at each time point in a durability monitoring study. We present the final protocol and justify our approach to establishing a standard methodology for durability monitoring of ITNs containing pyrethroid and a second AI.

Keywords: insecticide-treated nets (ITN); pyrethroid; mosquito; strain characterisation; insecticide resistance; method development; durability monitoring; product evaluation; quality control (QC); dual active ingredients (dual-AI); bioefficacy

1. Introduction

Insecticide-treated nets (ITNs) have been critical in controlling malaria. However, widespread resistance to the pyrethroids, which have been the sole insecticide class used on all ITNs until recently, threatens the continued effectiveness of standard ITNs [1]. Therefore, there is a need for new ITNs that include insecticides from classes with new modes of action to combat pyrethroid-resistant vector populations [2]. Several ITNs have been pre-qualified by the World Health Organization (WHO) containing a pyrethroid plus a second active ingredient (AI), which may be another insecticide (chlorfenapyr, CFPR; pyriproxyfen, PPF) or the synergist piperonyl butoxide (PBO), hereafter referred to as dual-AI ITNs [3].

There is a need to test the bioefficacy of ITNs in the laboratory. Here we are using the term 'bioefficacy' to mean the ability of a net sample to kill mosquitoes in a bioassay, contrasted with the efficacy of an ITN, which describes the net's ability to meet its objective of offering personal and community protection against transmission of mosquito-borne disease. Prototypes may need to be compared during product development, and research may be conducted to explore how an ITN works. Before distributing an ITN, the national malaria control programmes (NMCPs), or funders, may want to test its efficacy against local mosquito populations. During randomised control trials to determine the efficacy of ITNs (for example [4,5]), and during post-deployment monitoring (for example [6]), use and attrition of ITNs are monitored, and samples of deployed nets are collected over time to monitor their physical durability, analyse insecticide content and measure the bioavailability of each AI, using agreed-upon and validated bioassay methodologies (i.e., WHO cone bioassay or tunnel tests) [7,8]. This testing may be done at the time of collection, or all samples may be accumulated for simultaneous testing at the end of the study. Existing methods for durability monitoring [9], are optimised to evaluate pyrethroid-only ITNs, but the bioassay component may need to be adapted to be suitable for dual-AI ITNs.

The ability of a dual-AI ITN to kill insecticide-susceptible mosquitoes can be measured using standard methodologies and a susceptible laboratory strain. If the entomological endpoint of the second AI is different to the rapid knockdown and kill achieved by a

pyrethroid, it will be possible to separate the effect of the pyrethroid and the second AI. To monitor the persistence and additional efficacy of the second AI, a pyrethroid-resistant strain must be used, the majority of which will survive contact with the pyrethroid so that the effect of the second AI can be measured. Traditionally, mortality caused by pyrethroid exposure is measured to 24 h, as this insecticide class is fast acting. To control for delayed mortality caused by the pyrethroid in a resistant strain, where the second AI causes delayed mortality, mortality could be measured to the same time point when characterising pyrethroid susceptibility. The nature of the resistant strain needs to be considered, as this will affect the interpretation of data from the durability monitoring testing. The Vector Control Advisory Group (VCAG) of the WHO proposed the following criteria, in 2014, for mosquito strains suitable for use in screening for cross-resistance between insecticidal products [10]: at least 3 strains, two of which have significant metabolic resistance, representing the broad spectrum of known resistance mechanisms, ideally from a provided list of standard strains, or a strain that is fully characterised, and has a resistance level greater than 10-fold that of a susceptible strain of the same species at the LC_{50}, tested in parallel. Though not specified at the time, this LC_{50} would ideally be measured at the time point of interest for the second AI. This may form the basis of selecting suitable strains for bioefficacy testing or durability monitoring of dual-AI ITNs, but developments in the understanding of the molecular characteristics of mosquitoes have been made since these recommendations were released. New modes of action of insecticide are now being considered, meaning that there are limitations to these criteria, and practical challenges in meeting them, and they should, therefore, be reviewed.

The number of different resistance mechanisms that have now been identified, and would need to be screened to characterise a strain fully, is increasing over time and include overexpression of detoxifying enzymes [11,12], involvement of sensory appendage proteins [13] or the salivary protein gland [14,15], or cuticular thickening [16,17]. Insecticide resistant mosquito populations possess different combinations of mechanisms, and the relative contribution of these mechanisms to resistance differs between populations. These features have evolved to confer resistance to insecticides to which mosquitoes have been exposed, but some may also confer cross-resistance to new insecticides even with novel modes of action. Representing all known resistance mechanisms even in three strains, would be a major challenge, and, given that both the range of mechanisms expressed and our knowledge of these evolves over time, will always risk omitting resistance mechanisms that have not yet been identified. This is of particular concern for entirely new AIs coming to market, resistance mechanisms for which have not been identified.

Even if a list of standard representative laboratory colonies was established, there would be no guarantee of expected results in testing between sites or across time. For example, a colony that is nominally from a common strain may differ from a colony of the same strain held at a different test facility, due to differences in establishment and/or stabilisation in new laboratories and related selection pressures, genetic drift, inbreeding and genetic bottlenecks [18], insecticide exposure to maintain resistance [19] or through contamination events, rearing conditions that may affect fitness [20–22] or microbiome characteristics [23,24]. Resistance may shift over time, particularly if a strain is transferred between facilities or if selection pressure is not maintained. In addition, mosquito strains show temporal variability in their physiological response to insecticides. Routine efforts to characterise resistance phenotypes in lab strains are commonly based on the use of discriminating concentrations (DCs) or resistance intensity assays, rather than dose-response assays, which would be needed to establish LC_{50} values and resistance ratios.

Modes of action of insecticides used in dual-AI ITNs currently under evaluation mean that bioassays and protocols designed to measure bioefficacy of a pyrethroid may not be suitable. When considering chlorfenapyr, for example, the metabolic status of a mosquito is believed to affect metabolism of the pro-insecticide to the active form, and subsequently mortality rate [25]. Metabolic rate may be affected by extrinsic factors, such as temperature, time of day [26], and intrinsic factors, such as the nutritional status of the mosquito [27–29].

Bioassay choice and design may play a part, affecting, for example, how much mosquitoes move, or whether mosquitoes blood feed. As well as having higher rates of expression of detoxifying enzymes, different strains may have different metabolic rates, which may be related to the resistance mechanisms they express.

Finally, different criteria may apply when selecting a single strain or multiple strains to monitor the bioefficacy of dual-AI ITNs for durability monitoring, which is the focus of the present study. For example, to monitor the durability of ITNs it is not critical that the target species is used for bioefficacy testing, as long as the sensitivity of the species used is such that the bioefficacy of each AI can be detected across a relevant concentration range.

When conducting a durability study on ITNs, testing all samples at a similar time at the end of the study may help to minimise any temporal rearing effects on mosquito strains. Alternatively, testing ITN samples as they are collected avoids the logistical resources needed to store nets or net samples, and will minimise net degradation and/or loss of insecticide bioavailability before the bioassays. However, correct storage, according to manufacturers' instructions, should minimise degradation. Regardless of the approach, the large number of samples means it will not be possible for all net samples to be assayed by the same facility, at the same time, using the same cohort of resistant mosquitoes. In order to be able to compile and compare results of testing across a study, between facilities and time points, and to compare results between studies, there is a requirement for either (a) standardisation, such that the method and inputs are the same in all cases to minimise differences between results, or (b) characterisation of the inputs, so that results can be interpreted and, where differences are seen, any disparities between the inputs can be taken into account, or (c) a combination of the two. Depending on the specific questions of a study it might not be interesting to separate out the bioefficacy of each AI in a dual-AI ITN, and it may be sufficient to test the relevant endpoints in the mosquito population of interest or to compare results of bioefficacy testing with chemical analysis results on the same net samples. For the purpose of this consultation, we were interested in being able to separate out the effects of each AI, which is particularly relevant for randomised control trials (RCTs) of new types of ITN, where the durability of the second AI may not be known but is important to understand the added benefit over a pyrethroid-only ITN. In this case, the pyrethroid may be tested using a standard susceptible strain of the target species in the case where the second AI has an effect other than the rapid knock down and mortality caused by pyrethroids, but to test the additional benefit of the second AI (chlorfenapyr (CFPR), pyriproxyfen (PPF) or piperonyl butoxide (PBO)) mosquitoes must be pyrethroid-resistant, and assessed over the timescale of action relevant to the second AI. Inputs to the protocol for durability monitoring of dual-AI ITNs, therefore, include the pyrethroid-resistant mosquitoes used to test the second AI.

Selection, characterisation and standardisation of resistant mosquitoes are complex. The consultation process described in this paper aimed to produce a guide to the use of resistant strains for laboratory bioefficacy testing of dual-AI ITNs. Bioefficacy testing of ITNs tests for the presence of sufficient quantities of bioavailable compound to induce the desired endpoint in mosquitoes, usually mortality, and repeated testing over time can be used to measure durability of an ITN, for example, during an RCT trial. The purpose of this consultation was to develop, by consensus, a protocol for ensuring that the use of pyrethroid-resistant mosquitoes can be sufficiently characterised or standardised to allow compilation, comparison and interpretation of bioefficacy data across studies designed to monitor durability. A standard operating procedure (SOP) was produced which can be used by project teams, and is a supporting document to consensus SOPs recently developed for durability monitoring of new net types [30]. The same SOP may be useful in characterising a pyrethroid-resistant strain of mosquitoes for other purposes, or it could be adapted to meet different specific needs. This project forms part of a package of work to improve entomological methods in vector control, and is supported by Innovation to Impact (I2I) at the Liverpool School of Tropical Medicine (LSTM).

2. Materials and Methods

A group of experts was assembled, based on attendance at a preliminary discussion during a sidebar meeting at the American Society of Tropical Medicine and Hygiene (ASTMH) conference in November 2019, with additional invitees identified by the initial group, based on research interest in insecticide resistance, experience in the testing of new net types, or involvement in current or recent community scale trials of ITNs. Four virtual meetings of these stakeholders were held between April 2020 and August 2021, during which the need for a means to standardise or characterise resistant mosquitoes for the purpose of efficacy testing of dual-AI ITNs was agreed upon, possible approaches proposed and advantages and challenges of each discussed. Based on these discussions a protocol was drafted and iteratively refined by the group, who all then approved the final consensus SOP. A summary of these discussions, the final protocol, and the justifications for arriving at the proposed approach are presented here, and a detailed SOP is included as Supplementary Information.

3. Results

3.1. Possible Approaches to Achieve Standardisation or Characterisation

Several approaches to achieve either standardisation or characterisation were considered, outlined in Table 1. The advantages and disadvantages of each approach were discussed before a consensus approach was developed (Section 3.2).

3.1.1. Distribute the Same Well-Characterised Resistant and Susceptible Strains to All Test Facilities

Where bioassays need to be carried out at multiple facilities, one approach could be distributing a suitable resistant and susceptible strain to all facilities. Robust characterisation in the originating centre, and suitable quality control measures in receiving facilities, should remove strain differences as a variable in the assay. Strains could be maintained under the same selection and profiling regime, refreshed from a single facility if results of regular profiling start to differ, or refreshed every few generations from a single facility.

There are some practical limitations to this approach. The nature of a suitable strain may differ depending on the second AI under evaluation, and so this exercise may need to be repeated in parallel for each dual-AI ITN in a study. There may also be little benefit in terms of the workload of this approach over others. In the longer term, there are benefits to building the capacity of facilities to establish, maintain and characterise local strains. Regardless, this is the most straightforward approach to standardisation, provided the maintenance of the strain could be standardised between facilities.

However, there are two insurmountable issues. Firstly, it is unlikely that the same strain distributed and maintained in different facilities will remain static and comparable regarding its resistance profile. Even when under continued selective pressure, resistant phenotypes can shift over time [19,31,32], and there is likely to be a change in the resistance profile of strains associated with different genetic bottlenecks when moving between facilities, both as a result of small founding populations and as colonies adapt to their new environment. Even when rearing and selection are done under the same laboratory conditions, potential supplementary factors which are gaining more attention, such as the mosquito microbiome, may differ between insectaries and affect tolerance to insecticides [33–36]. Genetic drift will occur over time, even in well-controlled rearing facilities like the Malaria Research and Reference Reagent Resource Centre (MR4) at BEI Resources [37]. Changes in resistance can occur quite quickly [38]. So important differences may be missed before testing if the strain was not refreshed or re-characterised regularly to confirm that the resistance phenotype was still as expected.

Table 1. Possible approaches to achieve sufficient standardisation or characterisation of pyrethroid-resistant mosquitoes used for bioefficacy bioassays of dual-AI ITNs to compile, compare, and interpret results across studies. All approaches were proposed and considered by members of the stakeholder group and a consensus opinion reached as to their suitability and practicability; these are listed from most to least preferred or feasible approach.

Approach	Advantages	Disadvantages	Consensus Opinion
Characterisation of the resistant strain in parallel to the durability monitoring testing (see Section 3.1.3 below)	• Simple methods available to characterise phenotypic resistance to most AIs • Allows clear criteria to be set for a suitable strain • Generates useful information for interpreting testing results	• Standardised rearing and quality control measures also needed • Strains may still differ between test facilities, though within acceptable thresholds	Robust strain characterisation and quality control of mosquitoes ensure that mosquitoes are similar enough to compare data between test facilities and across time, and help to interpret inconsistencies. **Approach selected for further development into the final protocol.**
Sample and rear wild resistant populations for each round of testing and save samples for characterisation (3.1.4)	• Does not rely on all test facilities having suitable strains • May require less resources than maintaining strains long term • May be more predictive of local product efficacy; considers field-relevant resistance mechanisms	• Wild-caught mosquitoes highly variable, some characterisation recommended • Testing cohort might be a mix of species • Wild-caught mosquitoes less responsive in tunnel tests	Consistency of mosquito strain is important to detect any change in response to an ITN over time, not achieved by this approach. **Back-up solution to colony collapse or loss of resistance, rather than the primary approach to characterisation.**
Send all samples to several labs for repeat testing in a multi-centre study (3.1.6)	• No need to characterise mosquitoes • Consensus data is generated which may increase confidence in the result	• Higher testing workload, and each centre must maintain a resistant mosquito strain • Risk of ITN sample degradation during transport • Need for transport of samples between test sites	It is recommended as an additional step for quality control in a study to repeat testing on a sub-set of ITN samples at an additional site or sites. But costly and logistically challenging. **Not recommended as the primary approach to standardisation.**
Perform bioassays of nets from multiple time points side by side at the end of the study (3.1.8)	• Controls for variability in resistant mosquitoes over time and between test facilities	• Data not available until the end of the study, and real-time data are used to identify quality issues with ITNs • Large testing volumes • Risk of loss or degradation of ITN samples before testing	Repeat testing of a subsample of ITNs at the end of the study is recommended as a supplementary standardisation approach. Could test replacement ITNs to allow all ages to be tested in parallel. **Not suitable as a standalone standardisation measure.**

Table 1. *Cont.*

Approach	Advantages	Disadvantages	Consensus Opinion
Each test facility uses its own characterised resistant strain with a single standardised protocol (3.1.2)	• Conceptually simple additional step in method validation • Additional information about local mosquito strains • Capacity building from colonising and characterising strains	• Validation required against multiple strains (≥3), likely as a multi-centre study • Pyrethroid resistance varies between strains, complicating interpretation	Comparability between data collected at different facilities with different strains is a major issue. Unlikely that testing method would be robust enough to give consistent results between sites and across time points, regardless of the strain. **Not the preferred approach.**
Conduct all testing in a few chosen centres (3.1.5)	• Reduces the mosquito strains used in the study • Controls for other sources of variability between test facilities	• Delays caused by large testing volumes • Risk of ITN sample degradation during transport and storage • Little opportunity for capacity development	Unlikely to be an attractive solution for in country programmes of funders of durability monitoring studies. **Not the preferred approach.**
Measure the added effect of a dual-AI ITN relative to a pyrethroid-only net (3.1.7)	• Conceptually simple • Removes pyrethroid content as a variable • Controls for variability between strains or within a strain over time	• Vulnerable to changes in susceptibility to the second AI over time, and interaction between the two AIs in the formulation • Assumes lack of cross-resistance • Relies on the existence of suitable comparator pyrethroid-only ITN	Not sufficient as a standalone standardisation measure for durability monitoring. Including a pyrethroid-only net as a control is recommended, particularly if a suitable comparator is available. **Not the preferred approach.**
Distribute the same well-characterised resistant and susceptible strains to all test facilities (3.1.1)	• Straightforward standardisation • Only one strain needs to be characterised • Strain differences removed as a variable	• Validation needed for each dual-AI ITN • Colony resistance phenotypes and mechanisms may diverge after distribution • Biohazard risk in transferring resistant strains	Transporting insecticide resistant strains between sites within known or potentially habitable range of species is not acceptable due to biohazard risk. **Not feasible.**
Use a model system other than a conventional bioassay using mosquitoes of the target species (3.1.9)	• Could use a more amenable species, or very targeted or tailored approach • Could replace bioassays with a quicker, more robust method	• Equivalency would need to be established, and acceptability might be an issue	Further investigation to identify or develop new methods recommended for future use. **Suitable method not yet available.**

Second, and perhaps most importantly, because of the biohazard risk inherent in transferring resistant mosquitoes between geographical regions, there are strong reservations concerning this approach. There would need to be strict containment and quality control measures in place in all receiving facilities, but even then, there is a major ethical consideration in moving a strain that is potentially more resistant than the wild populations surrounding the second test facility. The MR4, for example, will perform case-by-case hazard assessments before distributing Anopheles strains and would not distribute strains where there is a risk of laboratory/insectary escape and potential for introduction establishment of a novel resistant population in a new environment [37]. In some situations, relevant parties may accept the idea, but the containment measures needed to make this approach safe may be too expensive or not feasible in practice. Alternatively, national, local or facility decision-makers may refuse to take on this responsibility and receive the mosquito strains. For these reasons, this is not a practical approach.

3.1.2. Each Testing Facility Uses Its Own Characterised Resistant Strain with a Single Standardised Protocol

Before a trial begins, the bioassay methodology used for bioefficacy testing, for example, as part of durability monitoring, should be optimised and validated using new and twenty times washed dual-AI ITNs with a susceptible strain (e.g., Kisumu). An additional validation step could be added with a range of different well-characterised pyrethroid-resistant strains to demonstrate that the method is not sensitive to differences in resistance mechanisms or population differences. In this context, a well-characterised strain would be one for which the phenotypic resistance profile was known, ideally with some understanding of the target site mutations, level of expression of detoxifying enzymes and other known mechanisms. Most test facilities that perform durability monitoring already hold pyrethroid-resistant strains that take some effort to characterise. They will often maintain them under selective pressure to preserve the resistant phenotype. There is a growing desire in the community to increase the capacity of local institutions, so this will increasingly be the case. Therefore, a pragmatic approach to standardisation could be that each facility uses a characterised local strain and relies on the testing methodology's robustness to give consistent results between facilities. This might be an attractive option to National Malaria Control Programmes (NMCPs) that would like to see data against mosquitoes that are closest in phenotype and genotype to the local mosquitoes that are responsible for malaria transmission.

If this approach were to be adopted as a way to compare results between sites, the method would need to be tested against a sufficient number of genetically heterogeneous strains, which would need to be sufficiently different for the validation to provide convincing evidence that the results would be comparable no matter what strain was used. There is some precedent. The WHO's VCAG have suggested three strains be used to screen for cross-resistance [10]. Phase II efficacy trials of ITNs require testing in an area with mosquitoes susceptible to all compounds in the ITN under evaluation, followed by testing in an area with pyrethroid-resistant populations [39]. However, it is unclear whether testing a single sample of nets against three resistant strains would provide sufficient evidence. This would be a significant burden in the efforts to validate a method. Assuming that containment facilities are not available, a single test facility is unlikely to have strains covering a broad geographical range. It is also unlikely that all resistance mechanisms will be represented by the strains the facilities maintain, thus necessitating a multi-centre validation approach.

This solution assumes that the testing methodology is sufficiently robust and specific enough and that the pyrethroid resistance is sufficiently high in the strains used that it is truly a test of the efficacy of the second AI alone. Since evidence for differences in resistance levels within the class is weak [40], characterising resistance to one example pyrethroid, or perhaps one representative Type I and one Type II pyrethroid, would be sufficient. However, even with very resistant strains, some individuals are usually killed

by exposure to pyrethroids, and mortality can vary substantially within, and between, bioassays [40]. So, some measure of the additional impact of the second AI is likely to be still needed, for example, a comparison to a pyrethroid-only ITN.

The group did not have confidence in comparability between data collected at different facilities with different strains. A bioassay is not likely to be validated sufficiently to give the same results, no matter the strain used for testing, because, for the following reasons, characterisation of strains will not be perfect: not all resistance mechanisms have been identified; those contributing most to resistance are not well understood; and not all markers are routinely screened for in all test facilities. There is evidence of this challenge in efforts by the WHO to set DCs for AIs by testing compounds against multiple strains of the same species and selecting a suitable dose based on the consensus of data [41]. The consensus opinion was that, although this is a pragmatic solution, the use of different strains with different resistance mechanisms and rearing methods are unlikely to give consistent results between test facilities or across time points, and so this was not the preferred option.

3.1.3. Characterisation of the Resistant Strain in Parallel to the Durability Monitoring Testing

The resistance phenotype of mosquitoes used for bioefficacy testing of dual-AI ITNs could be characterised by the following to ensure that they are suitable to effectively provide the information needed: sufficient resistance to pyrethroids, such that a high enough proportion survive exposure to the pyrethroid that the effects of the second AI can be measured, and susceptibility to the second AI. WHO tube bioassays to assess the susceptibility of the proposed strain to the WHO DC [42] of the pyrethroid, as well as the second AI included on the dual-AI ITN under evaluation, where a DC and method for evaluation are available, would be appropriate for this purpose; a straightforward and familiar method. Resistance intensity or dose-response assays with the AIs of interest would provide some quantitative information to help in defining a strain. A clear definition of a strain suitable for use in testing would be required, and the rejection criteria would need to offer a balance between pragmatism and the need for robust results.

Further characterisation could be done to further understand the strain and aid in the interpretation of results. This would require clear guidance on interpreting the bioassay results in the context of the strain characterisation. These could include, for example, DC assays with examples of type I and type II pyrethroids. All locally used insecticide classes in use for mosquito control could more fully characterise the strain's resistance profile. Testing for the presence of molecular markers associated with insecticide resistance would be informative, if the most informative or relevant molecular markers could be determined. This may not be practical on a routine basis, but strains held for bioefficacy testing would ideally be regularly screened for key molecular markers to provide a background understanding of the resistance profile of a strain and interpretation of data, e.g., response to PBO. In order to predict the efficacy of the product under evaluation, it would be helpful to confirm that a strain possessed key resistance mechanisms, against which the product under evaluation claims efficacy, and susceptibility to the second AI.

Beyond the resistance phenotype, there are multiple sources of variability between bioefficacy tests related to the mosquitoes used. Biological factors can affect observed levels of insecticide resistance, which may lead to differences between cohorts of mosquitoes from the same strain. For example, size [27], nutritional status [27,43,44], the temperature during rearing [28,45], and age [46,47], can all have an effect on mosquito fitness, and conditions during testing affect the results of bioassays [46] and references therein. Routine quality control and use of rearing SOPs (e.g., [19,48,49]) would be a robust method of ensuring that suitable mosquitoes are used throughout the study and across facilities and would ideally include fitness testing as a measure of the consistency of rearing methods and quality of the adults produced. When maintained in the absence of selective pressure, or selective pressure only from a single insecticide, resistance phenotypes and genotypes can shift in a laboratory colony over time [19]; regular selection for insecticide resistance should form part of a programme of quality control in maintaining a resistant strain of mosquitoes.

If a strain is intended for pyrethroid testing, it should be selected using a pyrethroid only insecticide, whereas a multi-resistant strain could be periodically selected with different insecticide classes, though this would be a significant undertaking.

While good rearing and testing procedures minimise most sources of variation [50], it would be informative to include some fitness testing (for example, wing length, average weight) of a sample of the cohort of mosquitoes used for bioefficacy testing, or, if possible, a sample from individuals which were killed and from those that survived characterisation or durability monitoring bioassays. A sample of each cohort of test mosquitoes could be stored for future analysis—for example, detailed characterisation of resistance mechanisms if results from one facility, or one-time point, varied from the others. This may be straightforward to compare changes in target site allele frequencies (e.g., kdr), but it may be more challenging for changes in metabolic gene expression, where defining a threshold of fold change is required, which would mean two populations were no longer comparable. Snap freezing at −80 °C would be ideal, so that relatively high yields of DNA and RNA can be analysed from stored samples, but even storage of dried individuals on silica would also be suitable for some further analyses.

If this approach were taken, differences in the resistance profile of strains used by different test facilities would still exist, and the strain or strains used may change in resistance phenotype during the study, but the robust characterisation and quality control should help to ensure that the key (known) parameters are similar enough, and that differences can be taken into account when interpreting data. This approach was selected for further development into the final recommended protocol. However, it was agreed that there would need to be a balance between the benefits of data robustness and the ability to reliably interpret results and compare across studies, and costs of additional workload required for extra bioassays and the ease of access to molecular characterisation.

3.1.4. Sample and Rear Wild Resistant Populations for Each Round of Testing and Save Samples for Characterisation

Where test facilities do not have access to a well-characterised resistant strain, or where issues, such as colony collapse or loss of resistance, result in non-availability of a suitable strain, a pragmatic alternative approach, sometimes employed, is to collect and rear wild resistant field populations for bioefficacy testing. Since wild-caught mosquitoes are likely to demonstrate large variability in the level of resistance and general robustness between collections, a cohort could be stored from each testing point for molecular characterisation. If sufficient material was available then phenotypic resistance and measures of fitness, including wing length, could be measured in parallel. While this approach still requires the team to have the capacity to maintain and characterise strains, less long-term commitment of resources may be needed, compared to holding strains over the whole course of the study. In some settings it may be very challenging to establish stable resistant colonies and using material maintained in the insectary for a generation or two to complete a study might be more practical. The biosafety concerns of transporting resistant mosquitoes between facilities can be avoided using local strains.

However, there would be a concern, especially when using F_{1s}, that testing is a mix of different species; this could complicate interpretation of results, power calculations, and assay replicate requirements. The storage of samples for later analysis would also help with this element of characterising the testing cohort of mosquitoes. If a colony could be established for later testing points in the study the strain could then be screened regularly and become more well-characterised.

For some purposes, using field-collected, or recently established, colonies of mosquitoes may be desirable. For example, it may be more predictive of field performance of an ITN than using established laboratory strains, since mosquito populations at different geographical sites may differ in their susceptibility to a given product [51], owing to the different resistance mechanisms they express, and potential for variability in levels of resistance across seasons [47]. Additional information would also be gained about the predicted

ongoing efficacy of the nets locally by using locally-collected mosquitoes for durability monitoring, which possess field-relevant mixtures of resistance phenotypes. This may be important for NMCPs when making ITN procurement decisions, though this may not be the case if recently caught wild mosquitoes are being mixed in culture with previously colonised wild mosquitoes. However, bioefficacy testing for ITN durability monitoring requires capacity to detect a change over time, so reproducibility of results and consistent longitudinal use of a well characterised strain is critical. If tunnel tests are required for testing of a dual-AI net (e.g., Interceptor G2) wild collected mosquitoes are unlikely to be suitable, due to low levels of attraction to guinea pigs, which often results in low levels of blood-feeding success in untreated control tunnels. For this reason, the group saw this as a backup option rather than the primary approach for using resistant mosquitoes as part of durability monitoring or similar study.

3.1.5. Conduct All Testing in a Few Chosen Test Facilities

Depending on the study design and available resources, it may be possible to standardise all bioefficacy testing by sending all sample ITNs to a single facility or to a small number of test facilities. In this way, the number of mosquito strains used across the study would be minimised, reducing variability between data sets. Other potential sources of variability are also controlled for, such as operator differences or the effect of different testing conditions. Comparing data between time points in a study would be easier than compiling data from multiple test facilities.

On the other hand, the need to test a large number of samples in a single test facility might cause a delay in processing the collected net samples, with the associated risks of changes to the resistance level of the mosquito strain between the start and the end of testing. Although net samples can be stored in refrigeration, there is also a risk of degradation during storage. This approach provides no control for the mosquito population changing between time points. Outsourcing testing to a single or small number of testing centres is unlikely to fit within country-specific National Malaria Control Program (NMCP) capacity development objectives. It may present challenges to the funders of durability monitoring studies. This was not a preferred approach.

3.1.6. Send All Samples to Several Labs for Repeat Testing in a Multi-Centre Study

Testing net samples in several laboratories could avoid the need to characterise mosquito strains in detail by testing the same samples against a different strain in each facility and evaluating result consensus by compiling the data, and assessing variability in results. Since the AIs may be unevenly distributed across a single ITN [52], giving different results from different samples of the same net, pieces should be cut along the same band to distribute to multiple facilities for parallel testing. Wherever possible, this testing would be done blinded.

This approach multiplies up the testing workload by the number of facilities. To reduce the additional workload, a study could circulate a sub-sample of net pieces to additional test facilities for confirmatory testing of the results obtained by the primary test facility, with careful consideration given to how to manage a situation where results did not match between facilities. Transporting ITN samples, particularly between countries, can be challenging. There is a risk of further degradation of samples, due to delay and during transport between facilities, and a requirement for each facility to colonise and maintain a resistant colony of mosquitoes. From a quality control point of view, it is good practice for a study to repeat testing on at least a subset of samples at different test facilities. It could be done intermittently as an additional level of quality control. However, from a logistics, and particularly from a cost, point of view, this would not be a feasible approach to standardisation for all studies.

3.1.7. Measure the Added Effect of a Dual-AI ITN Relative to a Pyrethroid-Only Net

Although it would always be beneficial to understand the nature of the strain of resistant mosquito being used for testing, an additional or alternative approach to measuring the bioefficacy of the non-pyrethroid AI is to simply expose them to both a pyrethroid-only net and the dual-AI ITN under evaluation, and use the difference in mortality between the two as the endpoint. This approach would allow the comparison of the additional mortality induced by the second AI between time points to be used as a measure of continued bioefficacy. Where the endpoint caused by the second AI is different to the mortality caused by the pyrethroid, for example in the case of PPF, which causes sterilisation, no correction is needed and the level of sterilisation is the measure of the bioefficacy of the second AI. Evaluation of this effect is only possible by using a highly pyrethroid-resistant strain so that sufficient mosquitoes survive exposure to the dual-AI ITN and can be scored for fertility. This is conceptually a simple and attractive approach, removing pyrethroid content as a variable and controlling for variability between strains or changes in a strain over time, at least in terms of the pyrethroid resistance phenotype. However, this approach assumes that tolerance of a strain to the second AI does not change over time, so that even if the strain changes in its pyrethroid resistance, its response to the second AI remains constant. It also assumes that there is no cross-resistance, i.e., that the mechanisms conferring resistance to pyrethroids do not also confer resistance to the second AI. Subsequently, if susceptibility to one AI changes over time susceptibility to the second AI remains unaffected. This may be true for some new insecticides, but there is evidence of cross-resistance mediated by cytochrome P450 enzymes [53,54], including between pyrethroids and pyriproxyfen [55], so it cannot be assumed.

If there is an interaction between the pyrethroid and the second AI in the formulated dual-AI ITN, then it may not be possible to make a straightforward comparison; the two AIs may not act independently, making a direct comparison between mortality on the pyrethroid-only versus the dual-AI net samples problematic. If it was possible to obtain comparable ITNs treated with each AI alone to compare bioefficacy of each with bioefficacy of the dual-AI ITN then a direct comparison could be made, and investigation of cross-resistance would be facilitated. On the other hand, the change in bioefficacy over time is relevant to a durability monitoring study. If the mortality caused by the pyrethroid-only net is sufficiently low it should still be valid to compare the additional mortality caused by the dual-AI ITN sample between time points.

Pyrethroid content is only removed as a variable if the pyrethroid-only net is equivalent to the pyrethroid content on the dual-AI ITN, in terms of the identity and concentration of the pyrethroid, as well as factors that might affect bioavailability, such as ITN formulation and impregnation method. For example, incorporated and coated nets may have different surface concentrations of AI and consequent bioavailability even where the total insecticide content is the same. This comparison becomes complicated for combination nets, such as the PermaNet 3.0, where the pyrethroid content is different on the roof and on the side panels. The selected pyrethroid-only net should be as close as possible in all characteristics to the ITN under evaluation, particularly for insecticide dose and bleed rate (where known). For some dual-AI ITNs no suitable pyrethroid-only net is available. A specifically matched pyrethroid-only net would likely rely on manufacturers producing small batches specifically for the purpose. This is not realistic, without incentive such as making it a requirement as part of the WHO Vector Control Product Prequalification (PQ) process, for example, and so the closest matching net would have to be used. The positive control should be kept consistent between time points; it may not be essential to be consistent between facilities if the relative change in additional mortality from the second AI over time is measured. A definition of 'brand new' or positive control net would be needed, along with guidance on storage conditions, especially for newer brands of nets, a method for washing and washing interval for the insecticide's regeneration.

Validation of the method against different second AIs using a range of resistant strains would be needed to have confidence in this approach, including the development of guidelines for the interpretation of results, establishing the threshold of killing when comparing the two nets, including the target minimum mortality among the resistant strain when exposed to the pyrethroid-only net, and gaining an understanding of the level of variability inherent in the assay. Additional controls could include exposing a susceptible strain alongside the resistant strain, including an unused and unwashed dual-AI ITN, or a net sample which only contains the second AI. However, this would likely have to be produced specifically for the strain characterisation by the ITN manufacturers, and again this is unrealistic without incentive.

It was agreed that this approach does not give sufficient standardisation for the durability monitoring studies under consideration. It might be enough for other purposes, such as screening field populations known to be resistant to pyrethroids to inform deployment decisions, but the variation inherent in these tests would likely lead to such wide confidence intervals in the data that it would not be sufficient for providing evidence to the WHO PQ Unit for vector control products assessment (PQT/VCP) of continued bioefficacy as part of durability evaluation in a product dossier. However, the consensus was that including a pyrethroid-only net in durability monitoring bioassays as a control would be good practice, if suitable net samples are available. The specific characteristics of the control net (brand, batch number, age, polymer, insecticide type and concentration, coated or incorporated, storage conditions) should be reported alongside the assay results. A pyrethroid-only control is not equivalent to the pyrethroid content or presentation in the dual-AI ITN. It could still be used as a proxy indicator to help calibrate and interpret test results, rather than an exact one measure to infer additional mortality induced by the second AI directly. The additional control of a brand-new dual-AI net would also be a way to control for the variability of the resistant strain over time, though with some of the same practical caveats as above. More generally, comparison between the bioefficacy of a pyrethroid-only ITN and a dual-AI ITN will help to inform procurement decisions.

3.1.8. Perform Bioassays of Nets from Multiple Time Points Side by Side at the End of the Study

To control for variability in the resistant mosquitoes used for testing over time or between test facilities, all nets sampled during the study could be stored and then tested in a short period at the end of the study. The major disadvantage of this approach is that information about the expected performance of the nets would not be gathered in real-time. Since durability monitoring is currently the main means of identifying quality issues with nets, this would have significant operational impact. There would also be the challenge of performing a large number of bioassays in a short period, rather than a smaller number at each time point, and the risk of a catastrophic event leading to loss of net samples from the whole study with no durability data being collected at all. Practical issues worthy of consideration are the potential for nets to degrade further during storage and the need for substantial storage space under specific controlled conditions. This approach to standardisation was agreed not to be suitable as a standalone standardisation measure.

A compromise would be to store a subsample of nets at each time point, after they have been collected back and used for bioassays, and repeat testing on this subsample at the end of the study, where resources allow. This has the advantage of confirming the results of bioassays conducted during the survey in side-by-side testing with minimal variation in the mosquitoes used, and could also be used to try to understand any unusual results observed during the study, supposing that data collected during the study were felt to be robust enough. In that case, a robust enough decision could be made to scale back this final testing or not continue at all, but the samples would be available as a backup. Additional standardisation measures would need to be taken during the initial bioassay testing performed during the study. Still, the group thought this could be a valuable addition to other characterisation or standardisation measures for WHO PQT/VCP studies, and monitoring of durability of nets in operational deployments. There is the opportunity

to build this into existing durability monitoring protocols used by PMI-supported studies and others [56], where nets are removed from use, typically within six months and annually for three years, for durability monitoring and replaced with new nets. Currently, these replacement nets are excluded from any further monitoring, but by the end of the study would represent nets of ages corresponding to each time point of the study and could be collected at the end for a final confirmatory round of bioassays. Important caveats of this approach include: there would still be a large amount of testing to be done at the end of the study, a more significant number of replacement nets would need to be distributed to account for attrition and leave a large enough sample for the final testing, and careful record-keeping would be required as different batch numbers may be distributed at other time points adding a layer of complexity. Nevertheless, the additional quality control of data could be used to justify the additional logistics and expense of this approach.

Iinformation would need to be generated on the likely variability between original and replicate testing inherent in the bioassay, so that the results of the repeat testing could be interpreted judiciously. Consideration should be given to how to report results of this repeat testing, particularly if initial monitoring data have been distributed or published already; protocols published ahead of the trial could make it clear that this repeat testing is part of the study design and careful interpretation and reporting of results which do not completely align will be required.

3.1.9. Use a Model System Other Than a Conventional Bioassay Using Mosquitoes of the Target Species

Conventionally, the durability of an ITN is tested using defined measurements of physical integrity, insecticide content and bioefficacy. For bioefficacy, cone bioassays, where the target mosquito species are exposed to a net sample, and the mortality is scored, is the accepted measure of field-collected ITNs over time [9]. Since the purpose of durability monitoring is to detect any change in bioefficacy of the net sample over time (i.e., ITN age), in a system that otherwise gives consistent results, the testing does not have to be against the vector species of interest. For bioefficacy testing in general, it is unnecessary to use the species against which a product will be targeted, as long as their relative sensitivities in a bioassay are understood. Aedes mosquitoes, particularly *Ae. aegypti*, can be reared in large numbers [57–60], with the added benefit of eggs resistant to desiccation, and can be stockpiled until sufficient eggs have been produced for a round of testing. It may even be possible to use a model organism, such as *Drosophila melanogaster*, to replace mosquitoes altogether, which has less of a containment risk and is easier to maintain, possibly expanding the number of test facilities able to perform durability monitoring. Validation would be needed to show that the chosen bioassay was appropriate for another species and that the species was sensitive to a change in AI concentrations across the relevant range. Even then, there may be reluctance to rely on results from a non-target species to test the efficacy of a product primarily aimed at anophelines.

New technologies are emerging which might offer a valid alternative to conventional bioassays or mosquito strains established from field-collected material. Transgenic strains of *Anopheles gambiae* over-expressing specific P450 enzymes, known to be important in conferring pyrethroid resistance, can be used to detect and characterise cross-resistance between insecticide classes [61]. A strain could potentially be produced that over-expressed the enzymes known to cause resistance to the second insecticide in a dual-AI ITN, expressing a very tightly defined resistance mechanism in a known genetic background.

Measures of bioefficacy suffer from high variability due to inherent bioassay variation and biological variation between mosquito populations. Chemical analysis of the total insecticide content of a net sample, for example, by HPLC, may be more reproducible but is not sufficient as a measure of bioefficacy, since it is the availability of biologically active insecticide on the surface of a net that determines its efficacy against mosquitoes [39]. However, suppose it was possible to sample and quantify the amount of bioavailable insecticide on a net surface. In that case, this might be quicker than performing bioassays

and an equally informative measure of bioefficacy. It would need to be correlated with the results of bioassays to be validated as a replacement method.

Novel techniques and approaches warrant further investigation, especially as ITNs continue to evolve. However, a method to monitor the residual bioefficacy of dual-AI ITNs is needed urgently, precluding much analysis of available options or the development of new systems.

3.2. The Final Protocol: Characterisation of the Resistant Strain in Parallel with Bioassays

The protocol for characterising the resistant mosquitoes used in bioefficacy testing with dual-AI ITNs is outlined in Figure 1, and a detailed standard operating procedure (SOP) is provided as Supplementary Information. The group agreed on this approach following several rounds of discussion on the merits of each of the proposed strategies and refinement of this preferred approach. The primary concern of the group was durability monitoring studies with dual-AI ITNs, but the protocol could be adapted to new types of ITNs as they are developed, to other product types, such as indoor residual spray (IRS) formulations, or attractive toxic sugar baits (ATSBs), and for other kinds of studies requiring resistant mosquito strains.

Since resistance changes over time, in both wild mosquito populations and laboratory strains, even when consistent selection pressure is applied, the only way to be confident in the resistance phenotype at the time of testing is to characterise the resistant strain simultaneously with bioassaying of the ITN samples. Depending on the study design, describing each cohort of mosquitoes used for bioassays on net samples could be laborious. Instead, a resistant strain could be characterised at the start and end of a study, for example, all net samples collected in a given year or from a given district. The following elements were considered to be key to the characterisation:

- The proposed colony of mosquitoes would be exposed to a discriminating concentration (DC) of the same pyrethroid as is present in the dual-AI ITN in a WHO tube assay to confirm their resistance phenotype. If the mortality was above 90%, the WHO definition of confirmed resistance [42], an alternative strain should be identified to complete the testing. Below this threshold, a strain with as low mortality as possible should be used to maximise the data generated to test the efficacy of the second AI.
- The proposed strain characterisation includes a PBO synergism assay to confirm susceptibility. Where a DC method has not been established or recommended by the WHO, and baseline susceptibility has not been demonstrated, some suitable method of exposure to the second AI should be included in the characterisation, and data monitored for changes in susceptibility over time. Mortality should be above 90% and ideally above 98%; values between 90 and 98% can be used to interpret the results of the main study.
- A PBO synergism assay is included in the proposed strain characterisation to confirm that metabolic mechanisms, most notably those associated with cytochrome P450 enzymes, are involved in the pyrethroid resistance of the strain used for bioassays. The group agreed that further investigation is warranted to determine what increase in mortality with pre-exposure to PBO indicates significant synergism, but suggested that the current WHO test procedures threshold of 10% is far too low to account for realistic variability in estimates [42]. Provisional analysis suggests that a mortality increase of 30% is required to provide meaningful evidence for impact.
- Standard untreated nets or solvent-only controls should be included in the assays used to characterise a resistant strain, with some additional controls. These represent a balance between gaining confidence in the assay and additional information against keeping the additional testing for strain characterisation to a manageable scale.
    a. A positive control brand-new pyrethroid-only net, containing the same pyrethroid content as the dual-AI ITN, should be included, for several reasons. Firstly, it provides an additional measure to ensure the strain has sufficient pyrethroid resistance, and will help interpret the results from the sample ITN bioassays.

Pyrethroid content is controlled for as a standard variable used to calibrate results between time points in the test net samples. Finally, exposing mosquitoes to a brand new pyrethroid-only net alongside the dual-AI test nets would demonstrate the added benefit of the second AI. Where multiple brands of such nets are available, the net most similar to the test net without the addition of the second AI should be selected, where possible of the same material and with the correct AI applied similarly (incorporated/impregnated) at the target concentration and release (bleed) rate. The brand should be consistent across the study.

b. Including a susceptible reference strain alongside the resistant strain acts as a control that the bioassay is functioning as expected, confirming the potency of WHO filter papers and pyrethroid-only net samples. It also serves as a benchmark to demonstrate the additional benefit of the second AI over that of the pyrethroid. Minimum mortality in the susceptible strain exposed to the DC of the pyrethroid in the dual-AI ITN and a brand new pyrethroid-only net should be 90%.

- An assessment of body size is included as a further quality control measure to help to interpret results with more confidence. Wing length is the recommended measure, but dry weight could be more practical. Size varies between species and rearing facilities, so it is not appropriate to set absolute thresholds, but collecting size data alongside the bioassay results is still valuable in helping to interpret bioassay results. For example, an unusually small cohort may explain anomalously high mortality in a bioassay [27].

Where both AIs induce mortality, the endpoint measures for the two AIs should be the same. If the outcome for the second AI is delayed mortality, then mortality caused by the pyrethroid should be measured for the same period. For example, in products containing chlorfenapyr, mortality is typically measured to 72 h [30,42], and so mortality should also be measured to 72 h for the pyrethroid treatments in the strain characterisation. This would control for additional delayed mortality in the resistant strain caused by the pyrethroid, which has been measured in some, but not all, strains tested [62–64], which could mean that for the purposes of the study the strain was not sufficiently resistant. Scoring knock-down and 24 h mortality for the pyrethroid exposure as well might be useful for comparison with historical data. If the second AI induces a different endpoint, for example sterility, and the study is aiming to measure the effects of each AI separately, then it would be necessary to include investigation of pyrethroid exposure on that endpoint.

A number of additional measures were recommended by the group as general good practice and to further characterise and standardise the resistant mosquitoes used for bioefficacy testing:

- It is desirable to use a strain with the same phenotype throughout a study. Efforts should be made to minimise heterogeneity of strain phenotype through time by standardising insectary rearing procedures, since insecticide susceptibility is affected by the size and general fitness of the cohort of insects used for testing. Standardisation of rearing conditions is especially important for strains used to test products such as chlorfenapyr, where metabolic activity is important in activating the insecticide and affected by rearing conditions and conditions during testing [25,65]. Although it may be unrealistic to ask for a single rearing SOP to be used between facilities, most facilities use some form of SOP to achieve standardised rearing and perform quality control (QC) measures, particularly those with GLP accreditation [66,67]. There are guidance documents available [48]. Some key considerations for maintaining the consistent quality of mosquitoes being reared for bioassays and steps taken to monitor quality are suggested in Table 2. Quality management systems help ensure that the data generated is reliable and reproducible and that it is possible to reconstruct a test in case there are any questions about data quality from manufacturers, regulatory authorities, etc.

- Strains are typically maintained under selective pressure and characterised routinely. These efforts could be enhanced by increasing the frequency of QC activities and including frequent selection and profiling with the pyrethroid of interest to the study. These data could be provided alongside the durability monitoring instead of parallel characterisation. Composite fitness indices can characterise mosquito populations used in experimental settings [68].
- If sufficient data for the colony exists, it is recommended to set upper and lower size thresholds, based on variability of size measured in the colony over a period of time, outside which testing would not proceed.
- The group recommended that a sample of mosquitoes be stored at the time of strain characterisation so that it could be characterised in more detail later if required to help explain an anomalous result, such as a drop in pyrethroid resistance or an increase in mortality from the second AI compared to a previous round of testing
- Inclusion of a brand-new dual-AI ITN of the same type as the test net samples as a control in the strain characterisation for durability monitoring studies would provide the following benefits (note that replicate pieces would be needed due to variation between and across an ITN):
    - Control for longitudinal variability in strains
    - Be a second measure of how much of the original bioefficacy has been lost over time in addition to the comparison between results obtained at the different time points
    - Allow the additional mortality caused by the dual-AI ITN over that of the pyrethroid-only net to be calculated at each time point and compared longitudinally
    - Control for any effects of declining content of the first AI over time. This is particularly important as the wash resistance of the pyrethroid and the second AI may be different, and so the additional benefit of the second AI may be lost before that of the pyrethroid
- Although a WHO tube assay is a standardised method to measure synergism, a cone test with a PBO net would provide a more realistic presentation of the PBO in combination with a pyrethroid because of simultaneous exposure. If a suitable PBO ITN is available as a comparator for the dual-AI being tested, for example DuraNet as a comparator for the DuraNet Plus or Olyset as a comparator for Olyset Plus, then cone tests with this ITN may be informative in more accurately evaluating the level of PBO synergism, and so metabolic resistance status of the resistant strain.
- SOPs will be required to collect, store, and transport net samples, both those tested and the control nets used for characterisation. The storage conditions and the maximum storage length are essential for incorporated nets. Typically, cut pieces are wrapped in foil and stored in a fridge. However, the development of these is outside the scope of this document.

It is strongly recommended that the results of strain characterisation be presented alongside study data to aid the interpretation of bioefficacy results. An example of how this might be done is shown in Table 3.

As an additional standardisation measure, the group proposes for durability monitoring bioassays that a sub-set of dual-AI ITN samples is retained from each study time point to repeat bioefficacy testing, and characterisation of the strain, with ITN samples from all time points in parallel at the end of the study. Suppose the nets are stored appropriately to minimise the degradation over time, in that case, this additional test allows for a direct comparison between samples to minimise the difference in the mosquito population and reconfirm the trend in mortality measured over time during the study. Since the bioassays were also performed during the study, a data set would still have been generated if storage conditions turned out unsuitable and samples were degraded, lost or damaged over time.

Table 2. Some key considerations for maintaining consistent quality of mosquitoes being reared for bioassays and conditions during bioassays, and steps that can be taken to monitor quality of mosquitoes.

Parameters to Standardise When Rearing Mosquitoes	Suggested Quality Control Processes in Mosquito Rearing	Parameters to Standardise When Performing Bioassays
• Temperature • Relative humidity (RH) • Controlled light/dark cycle • 1 h 'dawn' and 'dusk' • Larval density and feeding regime • Adult density in cages • Non-limiting access to a sugar solution	• Follow detailed rearing SOP • Routine monitoring of some fitness indicator/s [1] to follow colony health and rearing quality • Regular selection with at least one insecticide • Periodic profiling of resistance phenotype • Maintain staff training records on SOPs covering rearing and quality control • Equipment maintenance and calibration • Keep a record of deviations from SOP	• Temperature • Relative humidity (RH) • Time of day [2] • Feeding status (sugar, water, blood) • Age of mosquito • Measure a fitness indicator in testing cohort • Maintain staff training records on SOPs covering testing, data handling, archiving etc.

[1] In decreasing order of preference): composite fitness indices, wing morphometrics [69], wing length, dry weight, wet weight; [2] Mosquitoes may be reared on an adjusted light cycle to accommodate testing at a specific point in their circadian rhythm within working hours.

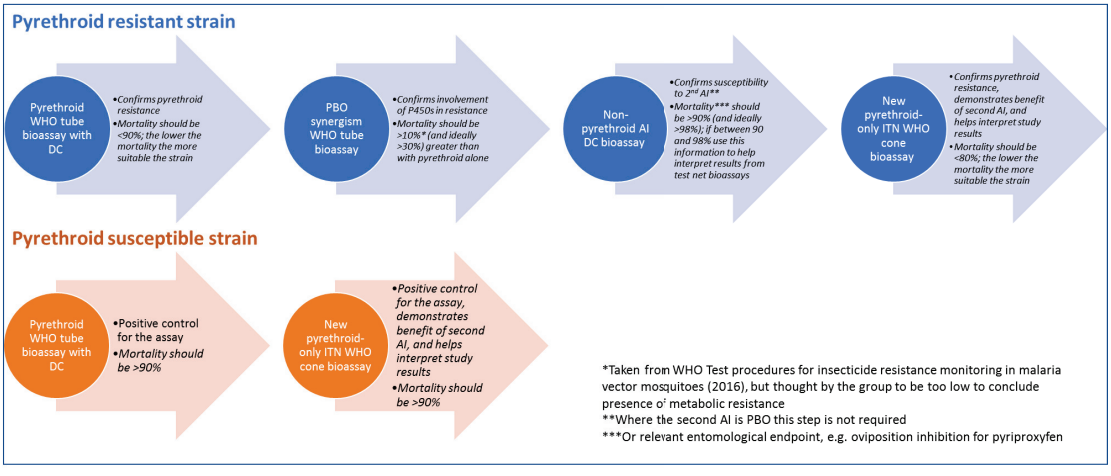

Figure 1. Overview of protocol for characterisation of a pyrethroid resistant strain for use in testing the bioefficacy of a dual-AI ITN, developed by consensus of a group of key stakeholders. Where delayed mortality (scored after more than 24 h) is the endpoint of interest for the second AI, mortality should be scored at this later time point for all elements of the characterisation; mortality may also be scored at 24 h.

An alternative to the retention and repeat testing of nets at the end of the study may be possible and has some advantages as an additional standardisation step. At each time point during a durability monitoring study, a sample of nets is collected from the field for destructive sampling (i.e., bioassay) and replaced with new nets of the same brand to prevent the household from being left unprotected. At the end of the study, a sample of these replacement nets could be collected alongside the nets being sampled for the final time point, and bioassays performed on all nets in parallel. In this way, nets of all ages could be tested side by side for a more direct comparison, with the same characterised strain of mosquitoes [56]. This approach avoids the risk of degradation of nets collected at each timepoint and held until the end of the study for parallel repeat

testing. A more complicated study design is required, and additional nets would have to be distributed to ensure sufficient nets remained at 36 months, since nets get discarded as they wear out. In carefully conducted research studies that employ unique labelling of individual nets, it should be possible if additional cost could be supported but not feasible for programmatic evaluations.

Table 3. Characteristics of a pyrethroid resistant and susceptible mosquito strain used for bioefficacy monitoring of dual-AI nets (an example is a strain used to monitor Interceptor G2, chlorfenapyr + alpha-cypermethrin ITN). Recommended format for presenting the results of strain characterisation should be provided alongside bioefficacy testing with dual-AI ITNs.

Pyrethroid Resistant Mosquito Strain: Tiassalé 13 **Species:** *An. gambiae s.l.*	
% Mortality (24 h) in WHO tube bioassay with alpha cypermethrin (0.03%)	54% (*n* = 94)
% Mortality (24 h) in WHO tube bioassay with alpha cypermethrin (0.03%) + PBO (4%)	92% (*n* = 96)
% Mortality (72 h) in WHO bottle bioassay with chlorfenapyr (100 ug/bottle)	100% (*n* = 97)
% Mortality (24 h) in cone test with new pyrethroid-only ITN (Interceptor)	70% (*n* = 106)
Pyrethroid susceptible mosquito strain: Kisumu **Species:** *An. gambiae s.l.*	
% Mortality (24 h) in WHO tube bioassay with alpha cypermethrin (0.03%)	100% (*n* = 90)
% Mortality (24 h) in cone test with new pyrethroid-only ITN (Interceptor)	95% (*n* = 103)
NB. All results Abbot's corrected where control mortality was between 5 and 20%	

3.3. Considerations and Points of Discussion in Deciding on the Final Protocol

3.3.1. Sample Size

When producing data to characterise a mosquito strain, the more mosquitoes tested, the more robust the result, achieved by increasing the number of replicate assays (cones, tubes or bottles). Given the inherent level of variability in the bioassays proposed, it would be desirable to recommend a minimum number of replicates on which a result should be based. The protocol proposed here uses the WHO test procedures for resistance monitoring [42] as a baseline measure of how many replicates are required for each assay, but as more data are produced using this protocol, more robust power calculations, or the application of modelling, can be used to refine the recommendation. However, if F_1 mosquitoes are used for testing upward-adjustment of sample sizes might be considered because of greater inherent variability compared to (inbred) laboratory strains [40], and is essential if species mixtures are expected.

3.3.2. Controlled Conditions during Characterisation of Strains

As with bioefficacy testing generally, it is necessary to control the climatic conditions during the strain characterisation bioassays. At minimum, the temperature, relative humidity, and time of day should be recorded and closely monitored in case of electricity cuts or other fluctuations. A reporting checklist would be helpful to encourage accurate reporting, whether the SOP is followed thoroughly or whether deviations have occurred for whatever reason. This will aid downstream interpretation of the results, and if temperature or humidity variation is implicated in production of apparently aberrant results, repetition of tests that were conducted out of specified ranges is advisable. Additionally, depending on the nature of the second AI, the time of day the bioassays, both strain characterisation and durability, testing are conducted might be critical [26,70–72]; for example, in evaluating a dual-AI ITN containing chlorfenapyr [25]. Some key parameters to consider standardising when performing bioassays with mosquitoes are suggested in Table 2.

3.3.3. The Approach Selected Must Be Applicable in Most or All Test Facilities

Proposed protocols must be practical, affordable, safe, and accessible in strain availability and facilities to maintain and characterise mosquitoes. The more criteria for suitable strains in place (multiple resistance mechanisms, resistance levels, characterisation methods), the more difficult it might be for test facilities to meet these criteria.

3.4. Deciding on Criteria for a Suitable Resistant Strain

The group's discussion over what criteria to set for a resistant strain was an attempt to strike a balance between a desire to characterise the strain in the greatest possible detail, to allow the best interpretation of data and comparison between data sets, and the pragmatic considerations of how much additional resource burden could be borne by programmes evaluating the dual-AI ITNs. The final criteria agreed by the group is highlighted in the protocol overview in Figure 1.

There was an agreement to recommend using a single well characterised strain for all testing within a study to remove this as a possible source of variation. ITN efficacy testing may be important to test against resistant strains of all significant *Anopheles* vector species, but this is not critical for durability monitoring. Indeed, the species used for the bioassays need not be a target of the ITN at all. As long as it is validated for the assay, it was sensitive to changes in bioavailable AI and relevant concentrations of the second AI.

There was a preference to use a strain with resistance conferred by multiple mechanisms, to produce the most widely applicable results; however, to confirm the presence of multiple mechanisms is complex and beyond the capacity of many test facilities. Although resistance to the first AI, currently always a pyrethroid, in the dual-AI ITN is the relevant requirement of the mosquito strain, and sufficient susceptibility to the second AI is required, a broader resistance profile might be desirable. It is likely sufficient to demonstrate resistance to the specific pyrethroid in the product under evaluation. Still, there may be a benefit to knowing that multiple resistance mechanisms are acting and using a strain shown to be resistant to pyrethroids in general and other insecticide classes. The consensus was that more information may always be desirable, and would help explain variable results across time or between test facilities. Still, an understanding of the resistance mechanisms present is probably not necessary for the question at hand.

Overexpression of cytochrome P450s appears to be the mechanism most commonly implicated in metabolic resistance and cross-resistance [53,56]. So, upregulation of P450s would be a desirable minimum criterion in a resistant strain. This could be demonstrated by characterising expression levels of a panel of key enzymes in the resistant strain (as detailed in [19]), and the potential for cross-resistance with the second AI of interest could be predicted with the use of P450 screens [54]. To adequately describe a strain's metabolic resistance risk, the most relevant molecular markers would need to be identified, along with the P450s most important in conferring resistance to the first AI, and then acceptability criteria based on fold-increase in expression relative to a susceptible strain would have to be established. This is challenging, however, and a strain showing a broad overexpression profile, including at least some known key enzymes (with proven insecticide metabolic capacity), may be more realistic. The interaction of P450s with the second AI would ideally be characterised as well. These analytical methods are specialised and relatively expensive, but regional reference laboratories may support programmes in analysing mosquito strains for this purpose. Given restricted resources, a programme could set out to molecularly-characterise the key strain, or strains, used for bioefficacy testing in durability monitoring at least at the start of the study. However, P450 expression levels are likely to change over time, particularly under selective pressure usually applied to laboratory-maintained resistant strains, so repeated analysis, perhaps of a reduced set of key markers or enzymes identified during initial characterisation, is desirable.

Given the costs associated with a more sophisticated analysis of resistance mechanisms, a pragmatic alternative is to demonstrate the involvement of metabolic resistance (primarily attributable to P450 enzyme activity) in the selected strain using a PBO synergism assay. Demonstrating that mortality is increased by PBO pre-exposure followed by a pyrethroid exposure relative to a pyrethroid alone may be sufficient to demonstrate the presence of P450-mediated resistance. This could be done with a WHO tube assay [42] or exposure to a locally relevant pyrethroid-PBO ITN, which would give useful efficacy data relevant to the local setting. Moving away from the standard protocol would make comparing facilities more challenging, though the standard synergism assay may not always be very

informative [40]. Testing with other synergists might be informative, as might testing the effect of PBO pre-exposure followed by exposure to the second AI. Still, standard methods have not yet been established [41].

The most pragmatic way to determine that a strain is suitable for monitoring the durability of the second AI in a dual-AI ITN is to confirm its resistance to the first AI, and ensure that it meets the acceptable criteria of mortality in a standard bioassay. This can be done through WHO tube bioassays using 1×, 5× and 10× DCs and selecting strains that are, for example, at least moderately resistant (<90% mortality at 5× DC) according to WHO definitions [42]. The resistance level could be determined more precisely using dose-response experiments to calculate LC_{50} values and resistance ratios relative to a susceptible comparator strain. If a standard SOP was used, these results could be compared between test facilities. Criteria that a minimum fold-increase in resistance be met before a strain was used for durability monitoring could then be set, though this is a labour-intensive approach, particularly since the LC_{50} for a susceptible population would ideally be set using multiple susceptible strains in a multi-centre study, to overcome the noise that is inherent in this approach. There may not be a need for strict resistance criteria since durability monitoring simply needs to detect a change in bioefficacy over time. However, a sufficient proportion of the exposed mosquitoes must survive exposure to the first AI to allow detection of an effect of the second AI.

It is important to measure the susceptibility of the resistant strain to the second AI in the product under evaluation in the absence of the first AI as part of strain characterisation. Even where an insecticide has previously not been used for mosquito control, resistance may have emerged as a result of agricultural use [2]. There is also the potential of cross-resistance to an insecticide with a different mode of action in mosquitoes resistant to pyrethroids, possibly through more general mechanisms that increase metabolism or reduce penetration. For example, the same metabolic enzymes appear to target pyrethroids and pyriproxyfen [56,57,73]. Programmes measuring the efficacy of a new vector control product should monitor the target population for emerging resistance. Still, it is also desirable to show that the resistant strain used to test the durability of the second AI does not already have a level of cross-resistance to it and that such resistance does not develop during the study. For PBO products this can be established during characterisation of pyrethroid resistance, as described above, and where the WHO recommends a DC and suitable methodology, this can be built into the strain characterisation [42]. Where such a method is not available for the second AI, cross-resistance may be predicted through molecular analysis [54], but this would normally need to be the subject of substantial additional investigation.

It is possible that the methodology selected for the bioefficacy component of durability monitoring could affect the criteria for a suitable resistant strain. For example, the cone test and tunnel test are very different modes of exposure and environments in which mosquitoes encounter a net sample for different exposure times and a strain that is not killed by the pyrethroid in an ITN in a cone test may be killed in a tunnel test. Where a non-standard methodology is used to measure bioefficacy, it is recommended that data from the baseline bioassays with a selected resistant strain be reviewed along with the data from the strain characterisation exercise to confirm that the strain and standard bioassays are suitable for that specific study.

3.5. Cost Implications of Adding Strain Characterisation to a Study

The development of this characterisation protocol seeks to outline an optimum method to characterise and standardise resistant strains for use in bioefficacy testing of dual-AI ITNs. These efforts are required to produce robust and reliable data, but additional funds will be needed to support the additional testing. Following the SOP detailed in Supplementary Information would add a workload consisting of six WHO tube assays with a pyrethroid, six WHO tube assays for the synergist experiment, six DC assays for the second AI and five cone tests with a brand new pyrethroid-only ITN with the resistant strain, plus two WHO

tube assays with a pyrethroid and five cone tests with a brand new pyrethroid-only ITN with a susceptible reference strain, a total of 475 resistant and 75 susceptible mosquitoes. If multiple types of dual-AI ITNs were included in a study, six additional DC bioassays would need to be added for each non-pyrethroid AI in the study, plus additional new dual-AI ITN positive controls, if included. The same mosquitoes can be used for QC and samples stored for later analysis, but these steps will require time commitment and consumables. Wing length analysis requires access to a microscope and image software or graticule, and further molecular analysis of samples may be required.

For a single experiment strain characterisation would be a one-time cost. Still, for a study lasting up to a month, strain characterisation should be completed before the study and repeated at the end of the study. Characterisation should also be repeated for longer studies, to ensure that the resistant strain has not changed in resistance profile and is still suitable, within one month of finishing, and where possible repeated during the study, on every mosquito generation if possible, or as often as practical. If resources were available to include some elements of the characterisation alongside each bioassay session, it would characterise the strain and provide an internal control for any differences between time points arising from changes in rearing, testing conditions, operator differences etc.

This additional cost may be small and easily borne for small scale research or development activities by academic institutes or developers or manufacturers of insecticide-based vector control tools. ITN evaluation and procurement is, however, a very price-sensitive market. Adding additional testing to the durability monitoring protocol will add cost to already expensive trials of new ITNs [74]. Durability monitoring is largely a donor-funded activity that is already growing in scale due to more complicated bioefficacy testing methodologies for dual-AI ITNs than was required for pyrethroid-only ITNs. The benefits of these additional characterisation steps will need to be accepted by funders, including the potential costs incurred should poor quality durability monitoring results lead to poor decisions on ITN choice. Decisions to procure more expensive ITNs can be made with greater confidence if the durability monitoring data are more robust in demonstrating their residual bioefficacy. Additionally, the scale of additional testing may be relatively insignificant compared to the bioefficacy testing already included in a study. For example, one reported durability study of ITNs in Madagascar required 50,000 mosquitoes to test 400 net samples [6]. The proposed protocol has been divided into minimum essential and additional desirable steps based on available resources. An exercise to calculating the cost of the characterisation and in scoping the willingness of funders to support may help promote the adoption of this proposed protocol. It is also likely that test facilities will support the minimum essential strain characterisation from multiple funding sources as it is incorporated into their regular facility running costs.

4. Discussion

Resistance to insecticides used to control mosquito vectors of disease is widespread, strengthening and evolving in the face of selection pressure from a limited number of chemistries available for use in public health [75]. New generations of insecticide-treated nets (ITNs) are now available based on novel mode of action chemistries, and other novel insecticide-based tools are in development to address this challenge. Dual-AI ITNs, including those containing two insecticidal compounds and a single insecticide paired with a synergist, promise greater effectiveness against pyrethroid-resistant mosquitoes. It is intended that the partner AI will have an effective lifespan of three years to match that of the pyrethroids currently in use, so that the new ITNs will fit into the existing campaign framework and contribute to resistance management. The dual-AI ITNs do, however, present a challenge in measuring their bio-efficacy in a laboratory setting, which is required to monitor their effective life through durability monitoring studies. Existing methods designed for ITNs containing only pyrethroids may not be suitable for those containing different modes of action insecticides or synergists. There is a need to test them against pyrethroid-resistant strains to quantify the entomological impact of the second AI.

Bioassays are an important proxy test for the surface availability of AI, and for demonstrating the efficacy of ITNs in killing mosquitoes under standardised conditions. As our understanding of resistance mechanisms increases, so does the complexity in determining relative contributions and how they affect the bio-efficacy of different chemistries and formulated products. These dynamics may alter with changing parameters, such as surface concentrations of AI declining over the lifespan of an ITN. There is, thus, the potential for the introduction of great variability into the results of bioassays designed for pyrethroid susceptible subjects, when considering the specific characteristics of the pyrethroid-resistant strain used, as well as methodological issues related to the mode of action of the ITN. To help minimise the noise in bioassay results due to these various factors, it is imperative that we clearly define or describe material inputs into these studies. A key aspect of this is to standardise or characterise the mosquito strains being used in these assays as far as possible, to provide interpretable data for analysis and to allow the comparison of results over time, between products and between testing centres. In an operational setting there is inevitably a need to balance improved characterisation or standardisation of inputs with the availability of suitable controls and logistical and financial constraints.

This paper describes a collaborative effort by researchers and implementers interested in insecticide resistance and evaluation of ITNs to agree on an approach to characterise mosquito strains to evaluate dual-AI ITNs and a set of specific criteria for the phenotype a suitable strain should have. Such an approach to method development, while somewhat time-consuming, does allow those implementing these activities to agree on a standardised method. This approach could be applied to other sources of potential variation in vector control efficacy and/or durability studies. For example, current guidelines for monitoring durability of ITNs were developed for pyrethroid-based nets but have been adapted and updated for dual-AI ITNs through a similar consensus approach by Innovation to Impact (I2I) [30].

Care was taken in designing a methodology for strain characterisation to ensure a comprehensive, robust approach, feasible in the context of the level of effort needed from those facilities carrying out this work. The standard operating procedure (SOP) decided upon and presented here (Supplementary Information) identifies some key parameters for characterisation, presents criteria for a suitable strain, and provides guidance on the rearing and quality control of the mosquitoes used in testing. Components are separated into those which are critical and those which are desirable and should be included where resources and logistics allow. Although these recommendations may have cost and time implications, these are balanced by promise of greater interpretability of the data produced in notoriously difficult studies to analyse and compare. The SOP will be made freely available through Innovation to Impact (I2I), to be trialled. Future studies with dual-AI ITNs, such as durability monitoring activities currently underway [76,77], allow its suitability to be reviewed and the methodology to be refined based on the experience of operators.

The consensus recommendation of the group of experts was to use a laboratory strain of mosquitoes for durability monitoring of dual-AI ITNs, to allow controlled rearing, quality control and characterisation to maintain and monitor the consistency of material over time. Regular and thorough characterisation of laboratory strains used for longitudinal bioefficacy testing is critical to ensure data validity and reliable interpretation of findings. There was some discussion favouring using locally relevant mosquitoes, and a desire to determine the operational significance of strain characterisation of bioefficacy data generated in the laboratory. Although the goal of durability monitoring is separate from efficacy testing, if the latter is a key question, there may be a preference for testing nets against local strains or against multiple recently-colonised resistant strains, which may express different, but locally-relevant, resistance mechanisms and give additional information about how the nets perform in situ. However, durability monitoring aims to determine that over multiple geographical locations ITNs continue to remain physically and biologically active for the duration of their expected lives. This is particularly difficult for dual-AI ITNs that must

be tested against resistant strains to ensure the non-pyrethroid component is still biologically active. For longitudinal experiments, such as durability monitoring or RCT trials, it is appropriate to use a well-characterised and consistent strain of mosquitoes. When a dual-AI ITN is being tested, it is critical to know the pyrethroid resistance phenotype of the mosquitoes being used. Positive control nets are a useful benchmark for interpreting changes in relative bioefficacy of dual-AI ITNs through time against a background of slight fluctuations in resistance phenotype of laboratory test strains.

The scope of this consultative exercise was the efficacy testing of the second AI in a dual-AI ITN combining a pyrethroid with a second insecticide or synergist. The 2022 Product Review Report from the WHO PQT/VCP team on insecticide treated nets formulated with a pyrethroid and either PBO or a second AI [78] recommended the development of 'improved guidance regarding the selection of mosquito strains to be used in bioassay and efficacy testing' including characterisation of resistance. The proposed strain characterisation approach addresses this need, and could be used in any situation where a pyrethroid-resistant strain is used in research. The general approach of characterising the biological material used in research and reporting results of the characterisation alongside the experimental data to aid interpretation is recommended as good practice. For example the WHO PQT/VCP Product Review Report [78] recommends the characterisation of the local vector population at the sites of experimental hut trials. This method establishes a solid framework that could be used with minor modifications to adapt to ITNs with unique AIs as they develop and become available. For example, specific additional or alternative considerations may apply when characterising a strain used to test ITNs containing two non-pyrethroid AIs. In this case, there will likely not be populations of mosquitoes available that are resistant to either AI. An alternative method would be needed to separate and measure the activity of each AI; for example, based on their differential speed of action. However, the requirements for maximising consistency and characterising the mosquito strain used to test the durability of these nets would be the same. The approach could also be readily adapted for characterisation of strains for evaluation of dual-AI products beyond nets, such as IRS formulations.

The development of this consensus methodology is part of a wider effort spearheaded by I2I to identify and address sources of variability in entomological data related to vector control product evaluation. To produce robust data, consistent across time and between operators, and to interpret results in a meaningful way, it is important to standardise or characterise material inputs into studies. The proposed method for characterising pyrethroid-resistant mosquitoes is the first of what is hoped to be a collection of supporting SOPs generated by, and made available to, the vector control community to help improve the generation and interpretation of entomological data for decision making.

5. Conclusions

To meaningfully interpret the results from bioassays and compare results between experiments it is important to maintain maximum possible consistency by standardising or characterising experimental conditions and inputs. When testing vector control tools, the target insect is a critical input. This work developed a method to characterise the resistance phenotype of pyrethroid-resistant mosquitoes used for bioefficacy testing of dual-AI ITNs. Adoption of this pragmatic yet informative approach will help in the interpretation of data from durability monitoring studies of these new net types. The approach can be adapted to characterise mosquitoes in other research involving biological materials where characterisation will help to generate consistent data which is more readily interpreted and compared, or where insecticide-treated materials are being used experimentally.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects13050434/s1: I2I-SOP-004: Strain characterisation of resistant mosquitoes for monitoring bioefficacy in ITNs treated with two active ingredients (Dual-AI ITNs) [79–81].

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Article

Colonization and Authentication of the Pyrethroid-Resistant *Anopheles gambiae* s.s. Muleba-Kis Strain; an Important Test System for Laboratory Screening of New Insecticides

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Simple Summary: Malaria control and prevention have traditionally relied on the use of insecticides in the form of treated bed nets or residual spraying in households. However, scaling up of these interventions—based on few available insecticide classes—resulted in the development and spread of insecticide resistance in malaria-transmitting mosquitoes. There is therefore an urgent need for introducing and applying new insecticides that are effective against these mosquitoes. Laboratories tasked with evaluating the efficacy of novel insecticides need to establish a large colony of resistant mosquitoes. In this study, we report the procedures used and challenges faced during the establishment and maintenance of a resistant mosquito strain in the laboratory which reflects the characteristics of the wild-resistant mosquito populations found in East Africa.

Abstract: Background: The emergence and spread of insecticide resistance in malaria vectors to major classes of insecticides call for urgent innovation and application of insecticides with novel modes of action. When evaluating new insecticides for public health, potential candidates need to be screened against both susceptible and resistant mosquitoes to determine efficacy and to identify potential cross-resistance to insecticides currently used for mosquito control. The challenges and lessons learned from establishing, maintaining, and authenticating the pyrethroid-resistant *An. gambiae* s.s. Muleba-Kis strain at the KCMUCo-PAMVERC Test Facility are described in this paper. Methods: Male mosquitoes from the F_1 generation of wild-pyrethroid resistant mosquitoes were cross-bred with susceptible female *An. gambiae* s.s. Kisumu laboratory strain followed by larval selection using a pyrethroid insecticide solution. Periodic screening for phenotypic and genotypic resistance was done. WHO susceptibility tests and bottle bioassays were used to assess the phenotypic resistance, while Taqman™ assays were used to screen for known target-site resistance alleles (*kdr* and *ace-1*). Additionally, the strains were periodically assessed for quality control by monitoring adult weight and wing length. Results: By out-crossing the wild mosquitoes with an established lab strain, a successful resistant insectary colony was established. Intermittent selection pressure using alphacypermethrin has maintained high *kdr* mutation (leucine-serine) frequencies in the selected colony. There was consistency in the wing length and weight measurements from the year 2016 to 2020, with the exception that one out of four years was significantly different. Mean annual wing length varied between 0.0142–0.0028 mm compared to values obtained in 2016, except in 2019 where it varied by 0.0901 mm. Weight only varied by approximately 0.001 g across four years, except in 2017 where it differed by 0.005 g. Routine phenotypic characterization on Muleba-Kis against pyrethroids using the WHO susceptibility test indicated high susceptibility when type I pyrethroids were used compared to type II pyrethroids. Dynamics on susceptibility status also depended on the

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lapse time when the selection was last done. Conclusions: This study described the procedure for introducing, colonizing, and maintaining a resistant *An. gambiae* s.s. strain in the laboratory with leucine to serine substitution *kdr* allele which reflects the features of the wild-resistant population in East Africa. Challenges in colonizing a wild-resistant mosquito strain were overcome by out-crossing between mosquito strains of desired traits followed by intermittent insecticide selection at the larval stage to select for the resistant phenotype.

Keywords: insecticide selection; out-crossing; strain authentication; laboratory screening

1. Introduction

Malaria vector control principally relies on the use of Insecticidal Treated Nets (ITNs) and Indoor Residual Spraying (IRS) as the most effective measures to prevent malaria transmission [1]. Historically, pyrethroids were used extensively for conventionally treated nets, superseded by Long Lasting Insecticidal Nets (LLINs), and also used for IRS due to their efficacy, relatively long persistence compared to other insecticides [2–4], and perceived low toxicity to humans [5–8]. However, the development and spread of pyrethroid resistance in malaria vector populations [4,9] demanded the development of new classes of insecticides with novel modes of action (MoA) for the control of mosquitoes and other disease vectors [10–13].

In developing new insecticides, several stakeholders are required in the process. The Innovative Vector Control Consortium (IVCC) has pioneered the bonding of prime agrochemical innovator industries, with research and academic institutions as key stakeholders in developing and evaluating new insecticides for mosquito control to prevent malaria and other neglected tropical diseases [14]. Research institutions perform laboratory and field screening of new chemistries for efficacy against mosquito populations and identify any cross-resistance risks at an early stage in the product development pipeline [15]; in this process mosquitoes are required as test systems [15,16]. In response to the global escalation of insecticide resistance in mosquito vectors, the WHO specifically recommends the establishment, authentication, and use of resistant mosquito strains during phase I efficacy testing of new non-pyrethroid insecticides [16]. This recommendation ensures that the evaluation will be able to capture efficacy against current resistance in malaria vectors. Authentication of a new insectary strain involves routine confirmation of the unique characteristics of the strain that sufficiently distinguish it from all others held in the same facility. This comprises routine validation of the species or subspecies identity, plus the resistance status as defined by genotypic and/or phenotypic characteristics [17,18]. In establishing a resistant insectary colony under artificial rearing conditions, the field sourced mosquitoes undergo several bottlenecks that could impair its suitability for the tests. Due to lack of variation and complexity in artificial rearing conditions, adaptation to these settings can favor populations to evolve in new directions from wild populations, especially when selection pressures and nutrition differ between the two settings [19,20]. Laboratory maintenance of insects in discrete generations facilitates selection for individuals that reproduce early and develop faster [21,22]. It is reported that adaptation to artificial environment can result in significant rapid evolutionary traits changing compared to natural populations [23,24]. This can lead to problems when reared insects are intended for release as biocontrol agents or in sterile insect control programs, when using laboratory strains to comprehend field population dynamics, and when using reared strains to predict vector control tools' effectiveness in the field. Attempts have been made to minimize the genetic drift and inbreeding effects through crossing an established laboratory stock with outbred field stock [25,26]. However, there is less utility for crossing the laboratory strain with field mosquitoes to maintain a complete genetic background of field populations when the colony is established to serve as a close representative for a few defined traits

which can be fixed, and when the ultimate use is limited to laboratory and semi-field environments.

In 2008, the Insecticide Testing Facility (ITF) of the Kilimanjaro Christian Medical University College-Pan-African Malaria Vector Research Consortium (KCMUCo-PAMVERC) Test Facility in Moshi Tanzania was initiated in parallel with a molecular laboratory, two insectaries, and three field stations. In the insectaries, the Test Facility established *Aedes*, *Anopheles,* and *Culex* mosquito colonies of different insecticide resistance profiles. From 2008–2011, *Anopheles* mosquitoes kept at the KCMUCo-PAMVERC Test Facility were limited to susceptible *An. gambiae* sensu stricto Kisumu (susceptible to all classes of insecticides used for vector control) and *An. arabiensis* collected from lower Moshi and reared to first filial generation (F1), the pyrethroid-resistant vector local to the Test Facility [27,28]. In 2012, the Test Facility acted to establish a colony of pyrethroid-resistant *An. gambiae* s.s. that would represent a typical East African resistant population. The *An. gambiae* Muleba-Kis strain was established and has been maintained in the insectary for years and propagated over hundreds of generations successfully, a feature emphasized by some scientists to qualify a colony as a strain [29]. The established *An. gambiae* Muleba-Kis strain is similar to East African *An. gambiae* s.s. populations for having the East African knockdown resistance (L1014S), a sodium channel mutation in *An. gambiae* that confers DDT and pyrethroid resistance [30]. The origin of L1014S mutation is Eastern Africa [30,31], hence the name *kdr*-east, although currently this mutation is no longer geographically restricted to East Africa [32,33] and its occurrence is frequently associated with the West African mutation L1014F [33,34]. Different types of pyrethoids, namely type I and type II, affect mosquitoes with *kdr* (East or West or mixture) differently. Pyrethroids are classified based on their chemical structures; type I pyrethroids lack the cyano-moiety present at the α position of type II pyrethroids. The type II pyrethroids generally delay the inactivation of the voltage-gated sodium channel substantially longer, and their effects are less reversible than type I pyrethroids [35]. Due to the similar steric profile with pyrethroids, DDT, an organochlorine, is affected with resistance to pyrethroids which often provides cross-resistance to DDT. A study by Reimer reported that mosquito populations carrying a high *kdr* frequency showed more resistance to DDT and type I pyrethroids than to type II pyrethroids [36].

The L1014S mutation has been fixed in a population of *An. gambiae* s.s. in Muleba District, north-western Tanzania [37,38], Busia, and Mayuge Districts in Eastern Uganda [39]. The occurrence of the L1014S mutation but at lower frequencies has been reported elsewhere in Tanzania [40], Kenya [41], and Uganda [42,43]. In previous studies done in Muleba district, where mosquitoes for this study were collected, it has also been reported that mosquitoes are resistant to bendiocarb, DDT, permethrin and deltamethrin, although there was no evidence for *Ace*-1 mutation [37]. Another study, a national-wide survey for resistance [44], reported *An. gambiae* s.l. resistance to pirimiphos-methyl for the first time in three sites (including Muleba district) out of 20 sites in Tanzania. Since the target site to organophosphates and carbamates is the AChE enzyme and that resistance in mosquitoes to this target site is frequently a G119S mutation in the *ace-1* gene [45], it is therefore reasonable to characterize L1014S and Ace-1 mutations as desired traits in the established colony to resemble the parental resistant population.

In this paper, we describe the procedures undertaken at the KCMUCo-PAMVERC Test Facility to establish a pyrethroid-resistant strain called *An. gambiae* s.s. Muleba-Kis. Here we focus on the procedures and lessons learned from out-crossing, artificial resistance selection, bioassays, and genotyping assays used to authenticate this strain for over two hundred generations. We describe data on the stability of resistance traits and fitness parameters over eight years. These data provide baseline resistance information on the outcome of the long-term intermittent selection of mosquito larvae.

2. Materials and Methods

2.1. Study Site

From April to May 2012, *An. gambiae* s.l. mosquitoes were collected in houses in two villages: Kyamyorwa (02°04′27.5″ S, 31°34′10.8″ E) and Kiteme (02°03′20.9″ S, 31°27′16.8″ E) in Muleba, a rural district on the western shore of Lake Victoria in northwest Tanzania (Figure 1).

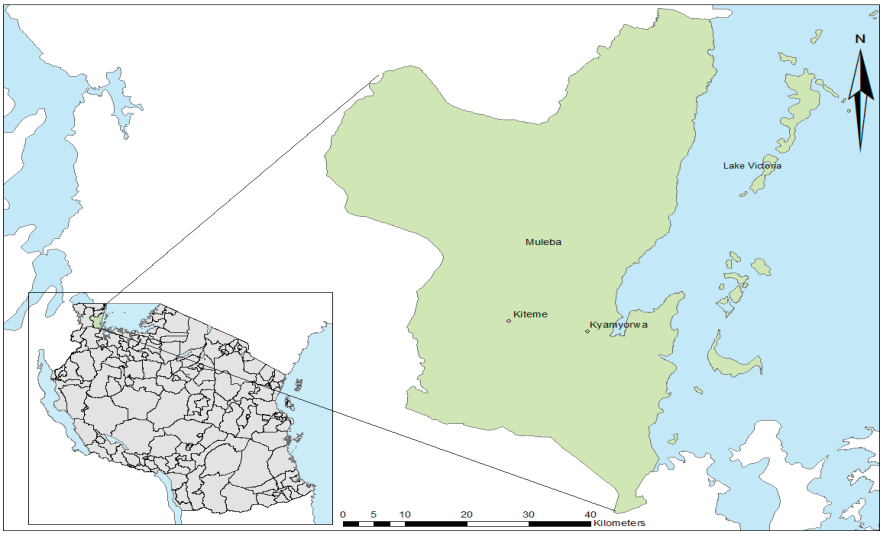

Figure 1. Map showing mosquito collection site in north-western Tanzania.

The mosquito collection for this study was part of an ongoing large cluster randomized trial in Muleba district, north-western Tanzania [46]. *An. gambiae* s.s. were the main vectors found in this area and have historically exhibited high resistance levels to pyrethroids [28], with mortality after exposure not exceeding 35%. The L1014S point mutation associated with pyrethroid resistance was nearly fixed, while no *Ace*-1 mutation was found [37].

2.2. Timeline

The timeline below, Figure 2, indicates the sequence of activities in this study across generations of *An. gambiae* Muleba-Kis.

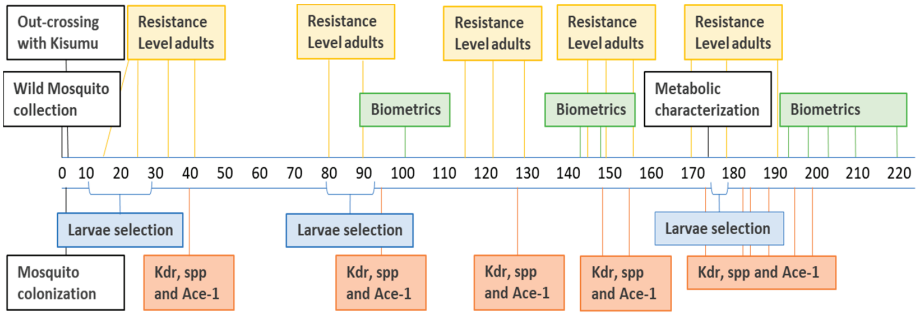

Figure 2. The timeline for activities, indicating wild mosquito collection, insectary colonization, out-crossing, selection, and strain characterization across *An. gambiae* Muleba-Kis generations.

2.3. Collection of Wild Mosquitoes and Introduction into the Insectary

Indoor resting blood-fed *Anopheles* were collected in house bedrooms using mouth aspirators. Mosquitoes were transferred in paper cups supplemented with glucose and transported to field insectaries located in Muleba. They were held under ambient relative humidity and temperature conditions in 30 × 30 × 30 mosquito cages containing a petri dish of moistened cotton wool overlaid with damp filter paper for egg laying. After laying, adult *An. gambiae* s.l. were stored individually and subsequently identified by Polymerase Chain Reaction (PCR) [47]. Collections were done over two months and eggs (approximately 500 eggs) were sent to the KCMUCo-PAMVERC Test Facility. Eggs were introduced into plastic bowls (6 L capacity) filled with 4 L of water. Larvae were reared under ambient temperature and relative humidity and fed with cereal for infants (Cerelac®, Nestlé Kenya Limited, Pate, Kenya) mixed with ground sardines at a 2:1 ratio. Adult mosquitoes were reared at 60–90% RH and 20–35 °C in cages (30 cm × 30 cm × 30 cm) covered with untreated netting material and provided with glucose solution 10%. To ensure optimal rearing conditions, insectary larval density was restricted to 200–300 per bowl (3 L capacity), water for mosquito rearing was pre-boiled to avoid bacterial infections, and environmental conditions (water and air temperature, relative humidity) were monitored and maintained.

2.4. Crossing for "Insectary Vigor"

When F1 mosquitoes were five days old, a restrained guinea pig was introduced into the cages of mosquitoes that were starved for one hour prior to blood-feeding. To overcome difficulties of adaptation to insectary conditions, out-crossing between female *An. gambiae* Kisumu and male *An. gambiae* Muleba mosquitoes were conducted. The main difficulties encountered were low blood-feeding, egg-laying, and survival, otherwise known as "insectary vigor." The *An. gambiae* Kisumu strain was obtained in 2008 through BEI Resources, NIAID, NIH: *Anopheles gambiae*, Strain KISUMU1, Eggs, MRA-762, contributed by Vincent Corbel. This strain is originating from Kisumu, Kenya, and was successfully established at our insectary and feeds well on guinea pigs. The Muleba and Kisumu strain pupae were collected separately, and males were separated from females on the first day after emerging. Adult male Muleba and female Kisumu mosquitoes were mixed at a ratio of 50:50 in a mosquito cage. These were reared at 20–35 °C, 60–90% RH, and a natural 12:12 h L:D photoperiod, and were provided with a guinea pig for blood-feeding and filter paper medium for egg-laying. This successful outcrossed mosquito was then named "*An. gambiae* s.s. Muleba-Kis strain" and has been reared at the KCMUCo-PAMVERC test facility since 2013.

2.5. Selection to Maintain Pyrethroid Resistance

In this study, selection was based on the exposure of larval mosquitoes to pyrethroid insecticides, and pyrethroids were chosen due to intensive usage in public health and having the most widespread resistance among mosquito vectors across Africa [48,49]. Artificial selection for pyrethroid resistance was started in the 15th generation. Six bowls each with around 100 larvae of 3rd to 4th instars were used initially, adopting a modified method by Shidrawi [50]. One mL of insecticide solution was added to 1 L of tap water at 27–32 °C, stirred for 1 min using a Pasteur pipette, and then left for 10 min to allow evaporation of acetone which was used as a solvent for insecticide solution preparation. Larvae were transferred into the glass bowl with the dissolved insecticide solution, each bowl with around 100 larvae. A small amount of larvae food was added and larvae were left for 24 h in the selection bowl. After 24 h, mortality was estimated. Mortality was estimated in three categories: high mortality, 67–100%; moderate mortality, 34–66%; or low mortality, 0–33%. The initial selection was done using permethrin, and later alphacypermethrin was used for colony selection. The initial permethrin concentration used for the section was 0.1 mg/L and increased to 0.2 mg/L at a time when larvae mortality was in a low category. The initial alphacypermethrin concentration was 0.025 mg/L and it increased to

0.05 mg/L when larvae mortality was in a low category. The larvae were sieved when still alive from the selection bowl, rinsed with 500 mL water (temp 27–32 °C) and returned to their original six bowls, and reared, while the dead larvae were removed. The selection was conducted intermittently. The availability of technical grade insecticide to make up the selection solutions and a need for mosquitoes for ongoing laboratory bioassays were the main constraints preventing routine artificial section of the colony.

2.6. Authentication of the Outcrossed An. Gambiae s.s. Muleba-Kis Strain

2.6.1. Phenotypic Resistance

WHO Susceptibility Test and CDC Bioassay

Insecticide susceptibility bioassays were done from the 17th to 196th generation, in accordance with WHO guidelines [51]. Bioassays were carried out using six insecticides, namely permethrin (0.75%), alphacypermethrin (0.05%), deltamethrin (0.05%), DDT (4%), bendiocarb (0.1%), and pirimiphos-methyl (0.25%), and tests were conducted at 25 ± 2 °C and 80 ± 10% relative humidity. Each type of insecticide bioassay was performed in 5 replicates, including one as a control. Twenty to 25, two-to-five-day-old female, blood unfed mosquitoes were tested, constituting a sample size of 100 to 125 mosquitoes for each insecticide. Tested mosquitoes were monitored for knockdown at 60 min and mortality at 24 h post exposure. In parallel with permethrin papers, limited WHO susceptibility bioassays were also conducted against bendiocarb papers (0.1%) and pirimiphos-methyl (0.25%). The insecticide resistance of the selected colony at the 190th generation was compared to the susceptible Kisumu strain using α-cypermethrin in CDC bottle bioassay [52] at concentrations of 52.5, 25.7, 12.5, 6.1, 3, 1.5, and 0 µg/bottle, where 12.5 µg/bottle acted as a discriminating concentration for *Anopheles* [51].

Synergist-Insecticide Bottle Bioassay

In a separate experiment (unpublished) in 2018, at the 143rd generation, a synergist assay with piperonyl butoxide (PBO) was undertaken to assess the role of elevated mixed-function oxidases in resistance. One hundred, 2–5-day-old female An. gambiae Muleba-Kis mosquitoes were tested for metabolic resistance using Piperonyl butoxide (PBO) in four replicates (25 mosquitoes per bottle) at a concentration of 100 µg/mL for one hour pre-exposure and then followed by 30 min exposure to permethrin (21.5 µg/mL), in accordance to the CDC guidelines [52], with the exception that mortality was considered at 24 h post-exposure. In brief, mosquitoes were pre-exposed to either acetone-coated bottles or PBO for 1 h at a temperature and humidity of 27 ± 2 °C and 70 ± 10% RH, respectively, during and after exposure. After pre-exposure, mosquitoes were transferred to holding cages for 60 min before being exposed for 30 min to bottles coated with either 21.5 µg/mL of permethrin or acetone as a control. After exposure, the mosquitos were transferred into holding cups and provided with 10% glucose-soaked cotton pads. After 60 min, post-exposure knockdown was recorded, and mortality was recorded 24 h post-exposure.

2.6.2. Genotypic Basis of Resistance

Detection of *kdr* and *Ace*-1 Mutations

The frequency of leucine to serine mutations (L1014S), termed *kdr* east (*kdr*-e) and *ace*-1, were assessed and frequently monitored using the method described by Bass et al. [47,53] to monitor progress in resistance development after successive insecticide selection events. For *Ace*-1 and/or *kdr*-e alleles, a total of 84–88 samples were analyzed by PCR per each test.

2.6.3. Species Identification and Biometric Measures for Fitness

Species Identification

To ensure colony species purity, at 43, 99, 131, 150, 162, 168, 178, 188, 190, 198, and 204th generations, the PCR for species identification was conducted using single nucleotide polymorphism genotyping [47]. At each generation, a total of 84-88 mosquito samples were tested.

Biometric Measurements

The size of individual adult females was estimated by the average length of left wings, while weight was measured by weighing the whole mosquito. To measure the wing length, a total of one hundred Muleba-Kisumu females were randomly sampled from five selected mosquito-rearing cages quarterly, covering the 99th to 204th mosquito generations. The wings were cut and placed on a stage micrometer (10 mm long with 100 × 0.1 mm (100 µm) divisions). Wing length was measured as the distance from the alula to the end of the wing where vein three ends [54–56] using an ocular micrometer at 2X objective magnification on a Nikon stereomicroscope, Model; SMZ 645 [Nikon Instruments, 1300 Walt Whitman Road, Melville, NY 11747-3064, U.S.A.], see Figure 3 below.

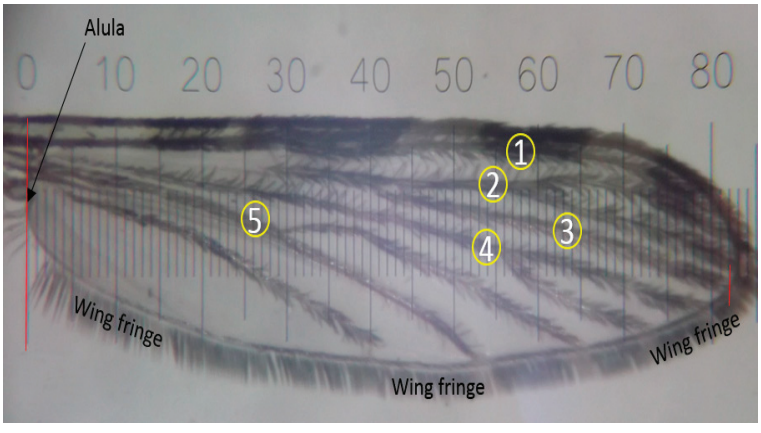

Figure 3. Image of wing aligned on a micrometer indicating the ocular gradations which correspond to the distance on the stage micrometer. Number 1–5 indicates the wing veins, where vein 3 is used for measuring the distance from the alula to the wing fringe (wing length). This photo was copied from PAMVERC Test Facility SOP with permission, originally taken and donated by MK (co-author).

Wing length and weight were continuously monitored in succeeding years regardless of whether selection with insecticide selection was done or not.

2.7. Statistical Analysis

The WHO criteria were used to classify the resistance or susceptibility status of the tested mosquito populations [51]. Descriptive analysis was performed to check for normality on wing and weight measures from the samples. Wing length and mosquito weight measures were all normally distributed. Using Stata [57], two sample T-tests were performed to compare wing length or mosquito weight across the years for the *An. gambiae* Muleba-Kisumu, and differences in mortality between *An. gambiae* Kisumu and *An. gambiae* Muleba-Kisumu across different concentrations in the CDC bottle bioassay.

3. Results

3.1. Colony Selection

Progressive selection with permethrin from the 15th to 29th generations for Muleba-Kisumu strain caused a drastic drop in susceptibility, indicated by the decrease in mortality (Figure 4). Inexplicably, although the same insecticide type and concentration was used for selection, susceptibility increased from the 30th generation to 35th generation. From the 35th generation, selection was performed using alphacypermethrin. However, the selection with alphacypermethrin was not associated with an abrupt decrease of susceptibility, as selection was infrequent.

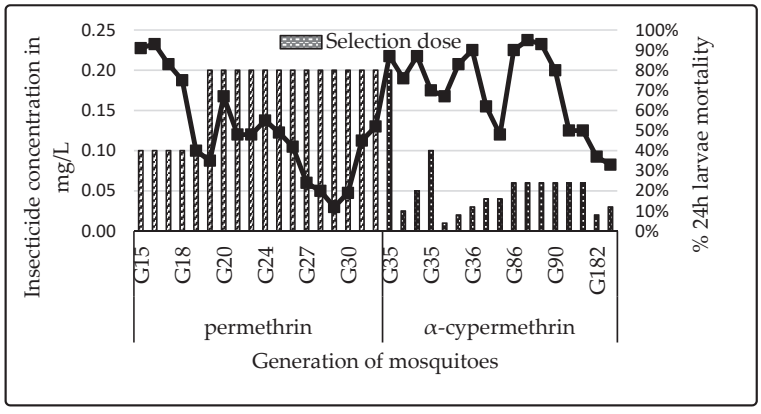

Figure 4. Dynamics of mortality rates of the selected larvae when different pyrethroids were used for the selection at different generations (G).

3.2. Phenotypic Resistance

3.2.1. WHO Susceptibility

The mortality observed in adult Muleba Kis exposed to permethrin (0.75%) test papers in the WHO susceptibility test was high (91% mortality) in the 17th generation (G17) and decreased to less than 20% at G25, then increased at G35. This follows a similar trend to the larvae mortality during selection procedures. The larvae selection with permethrin (pyrethroid types I) was not associated with a reduction in adult susceptibility when exposed to alpha-cypermethrin and deltamethrin (pyrethroid type II insecticides) using the WHO susceptibility bioassay at G35. Resistance to permethrin was the highest compared to the two other pyrethroids (α-cypermethrin and δ-methrin) from the 35th to 125th generations. In parallel with permethrin papers, WHO susceptibility bioassays conducted against bendiocarb papers (0.1%) and pirimiphos methyl (0.25%) resulted in 100% mortality, indicating that Muleba-Kis is fully susceptible to these insecticides. Mortality to DDT was consistently below 89% (Figure 5).

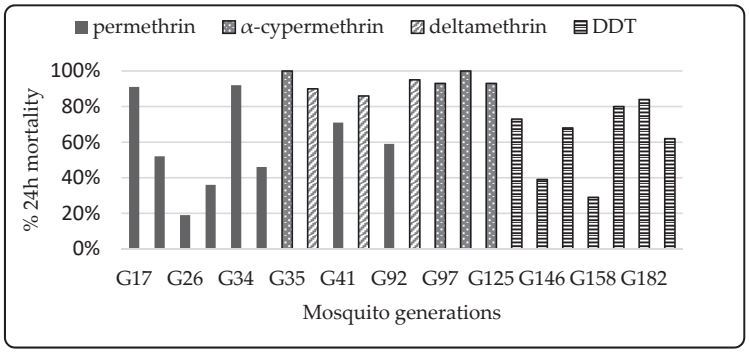

Figure 5. WHO susceptibility profiling of adult An. gambiae Muleba-Kisumu across generations. Mortality less than 90% indicates resistance, WHO (51). G = Generation.

On average, the Muleba-Kisumu strain's mortality was below the cutoff point (90%) when tested against permethrin (type I pyrethroid) and DDT papers. Only results with control mortality that were less than 20% were considered for analysis; tests when control mortality was higher than 20% were rejected. When tested against alpha-cypermethrin and deltamethrin (type II pyrethroids), the resistance level was low and above the cutoff value,

which is suggestive of susceptibility to this pyrethroid class. However, although several mosquito mortalities were above 90%, during the 35th, 89th, 97th, and 125th generations mortalities scored below 98%, which could imply existence of resistance.

3.2.2. Synergist-Insecticide Bottle Bioassay

CDC bottle bioassays were conducted with permethrin (PRM) and piperonyl butoxide (PBO) against a susceptible strain and a resistant strain. The susceptible strain showed >98% knockdown and mortality after exposure to permethrin, both with and without pre-exposure to PBO. Muleba-Kis showed resistance to PRM (73% mortality), which was restored to susceptible levels (94% mortality) after pre-exposure to PBO, indicating likely involvement of metabolic resistance mechanism in the *An. gambiae* Muleba-Kis strain; see Figure 6 below.

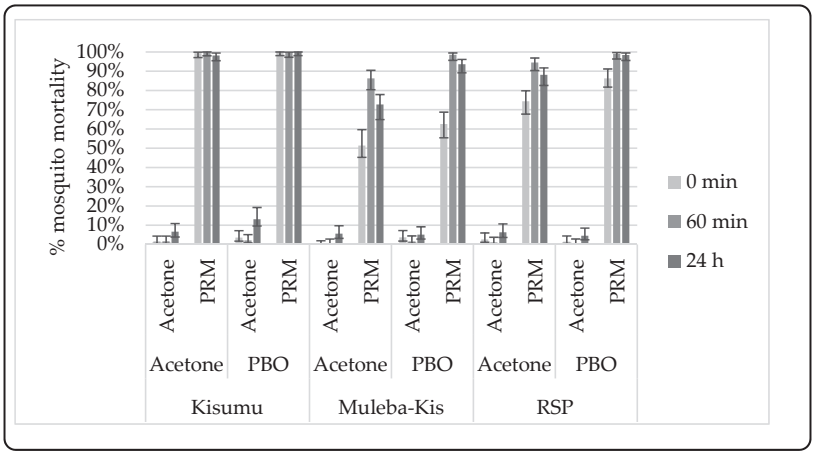

Figure 6. The knockdown and mortality rates of An. gambiae Kisumu and An. gambiae Muleba-Kis with and without PBO pre-exposure. Error bars are equivalent to 95% confidence intervals. PRM = Permethrin, PBO = Piperonyl butoxide.

3.3. Polymerase Chain Reaction (PCR) for Species Identification and Resistance Status

The *kdr* L1014S allele reached fixation in *An. gambiae* s.s. Muleba-Kis populations, coincident with the insecticide selection (Table 1).

Table 1. Molecular assays for Muleba-Kis strain over generations.

Generation	Number of Samples	Molecular Assay						
		Species		*kdr*-E			*Ace*-1	
		%Ar	%Ga	%RRe	%RSe	%SSe	%RRe	%SSe
G43	37	0	100	30	27	43	0	100
G99	57	0	100	100	0	0	0	100
G131	50	0	100	100	0	0	0	100
G150	84	0	100	100	0	0	N	N
G162	100	0	100	100	0	0	N	N
G168	84	0	100	100	0	0	N	N
G178	84	0	100	100	0	0	N	N
G188	84	0	100	100	0	0	N	N
G190	84	0	100	100	0	0	0	100
G198	88	0	100	100	0	0	N	N
G204	88	0	100	100	0	0	N	N

Note: Ar = *An. arabiensis*, Ga = *An. gambiae* ss, Ace-1 = insensitive acetylcholinesterase, RRe = homozygous mutant, RSe = heterozygous mutant, SSe = homozygous susceptible. When an assay was not done it is coded as N.

3.4. Resistance Strength of the Selected Colony: CDC Bottle Bioassay

Results from the CDC Bottle bioassay indicate that *An. gambiae* Muleba-Kisumu mosquitoes have lower mortality than *An. gambiae* Kisumu (Figure 7), which is suggestive of a higher level of pyrethroid resistance in the strain.

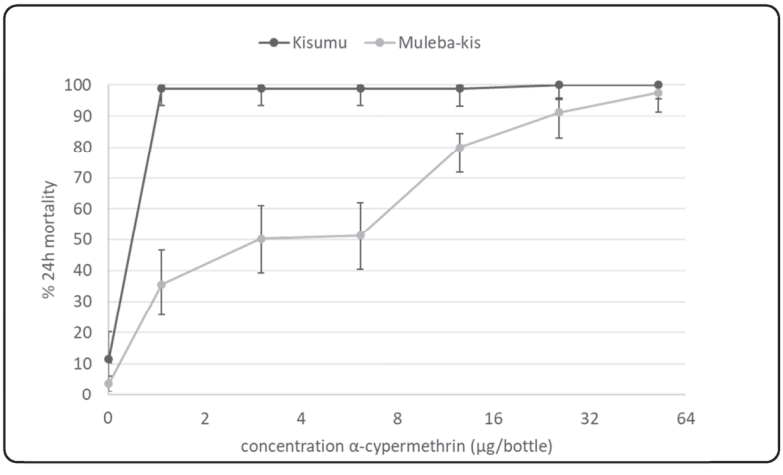

Figure 7. Mortality percentage of An. gambiae Kisumu and An. gambiae Muleba-Kis to varying concentrations of α-cypermethrin in CDC bottle bioassay.

At one and two times the diagnostic concentration of alphacypermethrin—12.5 µg/bottle and 25 µg/bottle, respectively—the Muleba-Kis strain showed significantly higher mortality than the Kisumu strain (two-sample *t*-test, $p < 0.001$). At four times the diagnostic concentration of the same insecticide—52.5 µg/bottle—there was no significant difference in mortality between the two strains.

Exposure of the Kisumu strain against alphacypermethrin in CDC bottles resulted in high mortality, indicating susceptibility against all doses, starting with a low dosage of 1.466 µg/bottle to the highest at 52.5 µg/bottle. On the other hand, exposure to the Muleba-Kis strain showed a dose-response, with mortality as low as 37% against the lowest dose and increasing to 98% mortality at four times the diagnostic dose.

3.5. Biometric Measures for Fitness

A total of 450 mosquitoes were analyzed: 50 in 2016, 150 in 2017, 150 in 2019, and 100 in 2020.

Data for female mosquito weight and wing length were normally distributed, hence we used the two-sample T-test to compare results between consecutive years. These results indicated that mosquito mean weight in 2017 was significantly higher than all other years, while the other years were similar to each other (Table 2).

Table 2. Dynamics in mosquito wing length across years 2016, 2017, 2019, and 2020.

Year	Samples (N)	Mean Wing Length	95% CI	*p*-Value *
2016	50	2.9504	2.8995-3.0013	0.6592
2017	150	2.9362	2.9035–2.9689	
2017	150	2.9362	2.9035–2.9689	<0.0001
2019	149	3.0405	3.0066–3.0745	
2019	149	3.0405	3.0066–3.0745	0.0025
2020	100	2.9532	2.9063–3.0001	

* Two-sample T-test.

On the other hand, mean wing length was only significantly higher in 2019 compared to the other years (Table 3).

Table 3. Dynamics in mosquito weight across years 2016, 2017, 2019, and 2020.

Year	Samples (N)	Mean Weight	95% CI	*p*-Value *
2016	50	0.0011	0.0010–0.0012	<0.0001
2017	150	0.0016	0.0015–0.0017	
2017	150	0.0016	0.0015–0.0017	<0.0001
2019	149	0.0012	0.0012–0.0013	
2019	149	0.0012	0.0012–0.0013	0.4281
2020	100	0.0012	0.0011–0.0012	

* Two-sample T-test.

4. Discussion

4.1. Blood-Feeding Challenges with Wild Mosquitoes

The propensity to feed on guinea pigs was not innate to the wild mosquito population and the colony could not be maintained by other means. The tendency to blood feed on guinea pigs was introduced by out-crossing, which is evidence for the genetic basis of intrinsic host-seeking factors within this Muleba mosquito strain. Host-seeking behaviors drive host choice, which is in turn driven by adaptive advantages that result from feeding on certain host species [58–60]. Wild mosquitoes were collected from bedrooms, which could indicate a preference of these mosquitoes to human blood. Similarly, observations from other studies [61,62] have associated host preference with the availability of host species for blood-feeding, which by their abundance form a readily accessible source of blood. This plasticity in host choice within mosquitoes could also be species- or strain-specific, accounting for differences in adopting a particular host as a blood source between different mosquito species or strains, as observed in this study where wild mosquitoes had a low affinity to guinea pig blood compared to the insectary-reared Kisumu strain.

4.2. Initial Low Insecticide Resistance Following Cross-Breeding

A common method used to establish resistant mosquito strain in the insectaries involves collecting wild-resistant mosquitoes and carefully maintaining them as they adapt to insectary conditions, usually going through a narrow bottleneck of few survivors in the first few generations. However, this endeavor has its challenges, such as failure of the wild strain to adapt to insectary temperature, relative humidity, and food; reduced mating; difficulties in blood-feeding on a new blood source; and reduced insecticide resistance. Early generations (15th to 17th) of Muleba-Kis strain in this study exhibited a low level of phenotypic resistance, which could be attributed to the low frequency of resistant alleles inherited from the resistant parent, the Muleba strain. The observed low frequency of resistant alleles, due to standing variation originating from the parental line before pesticide selection, is a phenomenon reported in other studies [63].

4.3. Impact of Mosquito Developmental Stage Used for Selection

The selection at the larval stage was chosen for three reasons. First, evolutionary pressure is strongest in young individuals to increase the probability of survival to reproductive maturity. Second, beneficial mutations at an older age can be associated with harmful effects in young individuals [64–66]. Third, by exerting the selection pressure to the aquatic stage of the mosquitoes, there is assurance for successive selection as it is impossible for larvae to survive subsequent selections but only through developing resistance [67]. Additionally, many reports have associated larvae exposure to trace amounts of pesticides with the development of insecticide resistance in malaria vectors [68–71].

In another study where larvae were selected, Shidrawi observed an increase of seven-fold resistance in an *Aedes* strain with initial moderate resistance when it was selected with DDT for eight generations [50]. When Shidrawi used different insecticides for the same

strain over a different selection period, he obtained a different resistance outcome. On the other hand, in a study where adult *Anopheles* were selected [72], using a pyrethroid type II in a period of a single generation the mortality level decreased from 42% to 18% over one generation, reflecting an approximately two-fold increase in resistance. Although these results indicate that adult selection induces a more appreciable increase in resistance over a short period when compared to the larval selection, further research is needed to correlate the two stages using the same strain of mosquito and the same insecticide. Additionally, since selection in this study used different insecticides in different generations, it is difficult to determine the period without selection which is taken to reverse resistance to full susceptibility.

4.4. Impact of Selection Using Pyrethroids

The resistance of the Muleba-Kis strain was based on a cross between the field *An. gambiae* s.s. from Muleba District (fixed for L1014S mutation) and the laboratory susceptible *An. gambiae* s.s. Kisumu strain, resulting in a weak resistance in an out-crossed F1 generation.

To overcome the problem of low resistance, the selection of insect colonies using a sub-lethal concentration of insecticide has been extensively adopted to increase or induce heritable resistance [73,74]. Several studies have successfully induced resistance by selecting either adult mosquitoes [50,72,75,76] or larvae [50,77–79]. Following the insecticide selection, a pre-existing low-frequency L1014S mutation became advantageous and was selected to a higher frequency in the population. Results further indicated that out-crossing between resistant and susceptible mosquito followed by positive selection has preserved the L1014S (*kdr*-e) allele inherited from the resistant parents, as similar results were obtained in other related experiments [80]. Likewise, Song and Leu [81,82] reported the gain of rodenticide resistance alleles by susceptible house mouse *Mus musculus domesticus* through hybridization with the intrinsically resistant Algerian mouse *Mus spretus*, followed by introgression under rodenticide selection. The increased insecticide resistance and affinity to guinea pig blood observed in the Muleba-Kis strain could have been inherited via a similar mechanism and is in line with the model for the inheritance of behavioral characters in mosquitoes [83]. However, intermittent selection might be the underlying reason for the observed small rises in susceptibility of the mosquitoes, as measured by WHO susceptibility tests. This reduced resistance due to withdrawal of selection is in agreement with other studies [72]. Apart from maintaining selection for resistance, currently there is no utility for crossing the Muleba-Kis strain to field mosquitoes to maintain a complete genetic background to field populations, as the colony was established to serve as a close representative pyrethroid resistant strain, fixed for the L1014S mutation intended for phase-I and Phase-II studies. However, when the colony is intended for field release, such as in male sterile technique programs or when used to comprehend field population dynamics, it becomes even more important to renew the colony with field material to address the genetic drift and inbreeding effects [25,26].

4.5. Differential Resistance to Type I and Type II Pyrethroids

Pyrethroids are classified into type I and type II based on their biological responses. While type I pyrethroids result in low kill with high recovery, type II pyrethroids result in high kill with low recovery. Type I pyrethroids bind preferentially to closed channels while type II binds to open channels [84]. Research has revealed that the level of resistance in houseflies with a *super-kdr* mechanism is below 100-fold for type I and is over 200-fold for type II pyrethroids [84]. Selection of the same mosquito strain could therefore generate different resistance outcomes depending on the insecticide type, class, and concentration used, among other factors. From this study, selection of larvae with pyrethroid type I correlated with increased tolerance to type I pyrethroid papers (permethrin 0.75%) in the WHO susceptibility test, and no significant tolerance was observed against pyrethroid type II papers (alphacypermethrin, deltamethrin) following the selection. A general observation from this study indicates that type I and type II pyrethroids cause different resistance

patterns, accounting for observed mosquitoes with less sensitivity to type I pyrethroids compared to type II pyrethroids. Similar results have been observed in other studies [85]. This variation is partly attributed to the different structural conformation between type I and type II pyrethroids that affect species selectivity and pyrethroid resistance [86]. Differences in structure and biological response between type I and type II pyrethroids are therefore presumed to be the underlying reasons for the different responses to selection observed in this study.

4.6. Metabolic Resistance

Although routine strain characterization by the WHO susceptibility test suggests that *kdr* was the underlying mechanism for resistance, limited PBO synergist bottle bioassay, which was done only once, indicated that mosquitoes' pre-exposure to PBO results in an increased susceptibility to permethrin by 20%, suggesting the role of metabolic resistance in this strain. However, the high susceptibility of this strain to bendiocarb and pirimiphos-methyl suggests a narrow role by metabolic resistance which requires more tests to confirm its contribution to an overall resistance. There is a need for testing for the gene expression levels, especially the CYP 450 genes which have widely been linked with metabolic resistance in malaria vectors across Sub-Saharan African [87].

4.7. Intermittent Quality Control Checks and Regular Strain Authentication

In this study, the quality of the mosquito colonies was checked to ensure that the rearing and selection procedures did not lead to contamination between strains or negative effects on the mosquito's weight or size. Underweight or undersized mosquitoes are not suitable for insecticide-testing assays, as they are more likely to be knocked down or killed by a given concentration of the insecticide. Furthermore, consistency of size is a good measure of the quality of rearing and helps to produce consistent and reproducible results provided that other rearing factors such as larval density, nutrition, environmental conditions, and microbial infection are controlled. The obtained results indicated that, despite out-crossing and insecticide selection of the strain, the weight and wing length remained fairly similar across the years, with the weight varying by only 0.001 g across four years, while wing length varied within 0.0142 mm and 0.0028 mm.

Contamination between strains held in the same facility is a regular error in mosquito rearing, especially when the same or closely related species are kept nearby [17,18,88]. The PAMVERC Test Facility keeps different strains of *An. gambiae* s.l. in different rooms and performs regular species identification using the PCR method [53] and resistance status checks to monitor for any cross-contamination. Results from characterizing the Muleba-Kisumu strain indicated that this species was identified as *An. gambiae* s.s. throughout the study, implying the absence of species contamination. *Anopheles gambiae* Muleba-Kisumu population was initially found to be partially resistant with only 30% having *kdr* fixed, but later *kdr* L1014S allele reached fixation in *A. gambiae* s.s. Muleba-Kisumu populations following the insecticide selection. These same populations exhibit strong degrees of phenotypic resistance to DDT and pyrethroid class I insecticides (permethrin).

4.8. Effect of Mosquito Weight and Wing Length on Phenotypic Resistance

Data for mosquito weight from 2016 to 2020 were normally distributed. The observed deviation in 2017 in mosquito weight could partly be attributed to changes in larvae food preparation. From 2016 to 2017, the preparation of fish flakes which are used as larvae food were microwaved at 150 degrees Celsius. However, this practice was terminated in 2018 as it was suspected to increase the nutrient content of larvae food. An increase in nutrient content or food is reported to lead to longer wings [89]. Results for median weight from 2016 to 2017 when there was no selection increased; from 2017 to 2019 weight decreased significantly; then from 2019 to 2020 the selection was ongoing and mean weight remained constant. The observed increase in weight before selection was mainly due to the nutrition regimen on the larvae. On the generations from 146–158, mosquito weight was

higher, with resistance thresholds equivalent to later generations (182th to 202th) when there was relatively low but maintained weight with ongoing selection. Maintaining the mosquitoes' weight is crucial, as it is the main determinant of insecticide susceptibility, and heavier mosquitoes are more likely to survive insecticide treatment [90]. Maintaining mosquito weight from year to year is essential in getting the correct interpretation from the WHO discriminatory concentrations [90], which is fundamental in both monitoring resistance development progress and strain authentication. On the other hand, mosquito wing length results were maintained except for 2019, where they were significantly higher relative to other years. Results obtained in this study indicate that progress and status of insecticide resistance are attributed to insecticide selection and are not confounded by weight or wing length. Furthermore, in this experiment there was a detectable difference between weight and wing length, however, there were no sufficient data to prove a direct correlation between wing length and mosquito weight. Although some studies [91] have observed a correlation between weight and wing length, other studies have reported a lack of correlation between wing length and weight [54,92].

5. Conclusions

Since its establishment, the PAMVERC Test Facility has played an important role as a key African research player in the chain of insecticide development, particularly in screening new active ingredients for mosquito control. Successful establishment of the Muleba-Kis strain in the insectary marks an important step in the colonization of a representative East African wild *Anopheles* population characterized with *kdr*-east mutation [30]. This insectary colony enables the evaluation of vector control tools under the current East African insecticide resistance challenge. This study has also demonstrated that blood-feeding failure and low insecticide resistance in colonized mosquitoes can be overcome by out-crossing desired traits between mosquito strains followed by intermittent insecticide selection at the larval stage. It is worth mentioning that, with the interest in developing and bringing new insecticides into the market, it is crucial to quantify the fitness cost associated with resistance [93], and that although our test facility has managed to successfully create a resistant line through the described methodology with comparison to cited separate studies, further research is needed to perform a direct comparison between various selection methods and to assess the level and duration it takes to establish resistant lines in the same mosquito strain. The capacity to establish resistant mosquitoes allows for assessment of new insecticides for efficacy, cross-resistance, and the likelihood of resistance development to a novel insecticide, therefore providing an early alert to plan for an effective pre-emptive resistance management program.

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Sympatric Populations of the *Anopheles gambiae* Complex in Southwest Burkina Faso Evolve Multiple Diverse Resistance Mechanisms in Response to Intense Selection Pressure with Pyrethroids

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Simple Summary: Targeting mosquitoes with insecticides is one of the most effective methods to prevent malaria transmission. Although numbers of malaria cases have declined substantially this century, this pattern is not universal and Burkina Faso has one of the highest burdens of malaria; it is also a hotspot for the evolution of insecticide resistance in malaria vectors. We have established laboratory colonies from multiple species within the An. gambiae complex, the most efficient group of malaria vectors in the world, from larval collections in southwest Burkina Faso. Using bioassays with different insecticides widely used to control public health pests, we provide a profile of insecticide resistance in each of these colonies and, using molecular tools, reveal the genetic changes underpinning this resistance. We show that, whilst many resistance mechanisms are shared between species, there are some important differences which may affect resistance to current and future insecticide classes. The complexity, and diversity of resistance mechanisms highlights the importance of screening any potential new insecticide intended for use in malaria control against a wide range of populations. These stable laboratory colonies provide a valuable resource for insecticide discovery, and for further studies on the evolution and dispersal of insecticide resistance within and between species.

Abstract: Pyrethroid resistance in the Anopheles vectors of malaria is driving an urgent search for new insecticides that can be used in proven vector control tools such as insecticide treated nets (ITNs). Screening for potential new insecticides requires access to stable colonies of the predominant vector species that contain the major pyrethroid resistance mechanisms circulating in wild populations. Southwest Burkina Faso is an apparent hotspot for the emergence of pyrethroid resistance in species of the Anopheles gambiae complex. We established stable colonies from larval collections across this region and characterised the resistance phenotype and underpinning genetic mechanisms. Three additional colonies were successfully established (1 An. coluzzii, 1 An. gambiae and 1 An. arabiensis) to add to the 2 An. coluzzii colonies already established from this region; all 5 strains are highly resistant to pyrethroids. Synergism assays found that piperonyl butoxide (PBO) exposure was unable to fully restore susceptibility although exposure to a commercial ITN containing PBO resulted in 100% mortality. All colonies contained resistant alleles of the voltage gated sodium channel but with differing proportions of alternative resistant haplotypes. RNAseq data confirmed the role of

P450s, with CYP6P3 and CYP6Z2 elevated in all 5 strains, and identified many other resistance mechanisms, some found across strains, others unique to a particular species. These strains represent an important resource for insecticide discovery and provide further insights into the complex genetic changes driving pyrethroid resistance.

Keywords: malaria vector; insecticide resistance; insecticide treated nets; cytochrome P450s; kdr; cuticular resistance

1. Introduction

Pyrethroid insecticides have played a key role in interrupting malaria transmission. All insecticide treated nets (ITNs) in use contain pyrethroids; they are the major active ingredient in insecticidal household aerosol sprays and coils and, prior to the advent of widespread resistance, they were the preferred chemistry for use in indoor residual spraying programmes [1]. Malaria vectors will also likely encounter pyrethroids in their aquatic habitats as this insecticide class is still widely used in agriculture, and mosquito breeding sites in rural areas frequently contain detectable levels of insecticides utilised to spray nearby crops [2,3].

Resistance to pyrethroids was first detected in African malaria vectors in the 1970s [4] and is now widespread [5], prompting the search for new chemistries for use in vector control tools. Whether re-purposing chemistries used to control other pest species, or searching for new insecticide classes, the identification of suitable chemistries requires a robust screening pipeline that includes screening potential compounds against a range of mosquito populations resistant to current chemistries [6,7]. Whilst ultimately testing against natural wild populations will be required, the availability of stable laboratory colonies of the predominant vector species, containing the major resistance mechanisms circulating in the field can greatly accelerate the insecticide screening pipeline by identifying resistance liabilities at an early stage [8].

We have previously described the properties of several colonies of *Anopheles* mosquitoes that have been widely used in insecticide discovery programmes; these contain well characterised target site mutations and metabolic resistance conferred by elevated levels of specific pyrethroid metabolising cytochrome P450s [9]. However, recent studies on *Anopheles gambiae* s.l. mosquito populations from West Africa have identified additional, potent pyrethroid resistance mechanisms such as reduced penetration caused by cuticular thickening [10,11], insecticide sequestration by pyrethroid binding proteins in the mosquito appendages and novel resistance associated haplotypes of the pyrethroid target site, the voltage gated sodium channel (VGSC) [12–14]. Several of these resistance mechanisms could potentially cause cross resistance to existing or new classes of insecticides; thus, we sought to establish new colonies of pyrethroid resistant *An. gambiae* s.l. from Burkina Faso, stabilise and quantify their pyrethroid resistance phenotypes and determine the underpinning mechanisms responsible for resistance.

An. gambiae is a species complex of at least nine morphologically identical species. Three of these (*An. gambiae* s.s, *Anopeheles coluzzii* and *Anopheles arabiensis*) are amongst the most important malaria vectors and are found in Burkina Faso [15]. Introgression of genes under selection pressure is not uncommon between members of the complex with several well documented cases of exchange of haplotypes containing point mutations in insecticide target sites [16,17]. The Southwest region of Burkina Faso is an important agricultural region of the country and also an area of stubbornly persistent malaria transmission, perhaps partially linked to the exceptionally high levels of pyrethroid resistance in the malaria vectors from this region [18,19]. We established three new colonies from larval collections in the Cascades and Southwest regions of Burkina Faso between 2015 and 2018, encompassing each of the three members of the *An. gambiae* complex found in the country. Phenotyping and molecular characterisation of these new colonies, the previously established Banfora

M colony (Cascades region) and the VK72014 colony (neighbouring Hauts Basin region), revealed high levels of pyrethroid resistance with four colonies meeting the WHO definition of high intensity resistance and the fifth with moderate intensity. Genotyping and RNAseq identified resistance mechanisms in common between strains but also key differences that may have implications for susceptibility to alternative insecticide classes.

2. Materials and Methods

2.1. Establishment of Strains

Details of the strains used in this study are provided in Table 1. The origins of the susceptible strains Kisumu and Moz and the pyrethroid resistant Burkina Faso populations VK7 2014 and Banfora M have been described previously [9]. Larval collections from multiple villages in the Comoé Province, Cascades region of Burkina Faso in 2015 led to the establishment of two strains: Bakaridjan and Banfora. Briefly, larvae were reared to adults, allowed to mate and then females transferred to Eppendorf tubes to oviposit individually as described previously [20]. Females were killed by freezing after oviposition. Dried females, and egg papers were transported to the Liverpool School of Tropical Medicine. Species ID on the F0 female was performed [21] and egg batches from *An. gambiae* (s.s.) or *An. coluzzii* females were pooled to establish two separate colonies. The *An. coluzzii* colony was named 'Banfora' after the Banfora district as the colony was established from collections from several villages within this district (Tiefora, Pont Maurice, Sikane and Djomale; Figure 1). The *An. gambiae* s.s. strain was named 'Bakaridjan' as the majority of egg batches used to establish this strain were collected from this village. The *An. coluzzii* Tiefora strain and the *An. arabiensis* Gaoua-ara strains were established as above from larval collections performed in Tiefora Village Comoé Province, Banfora District and Gaoua District, Poni Province in 2018. The insecticide-susceptible colony N'Gousso originated from Cameroon [22].

2.2. Mosquito Rearing

Insectaries were maintained under standard conditions at 26 ± 2 °C and 70% relative humidity ±10% under L12:D12 h light:dark photoperiod. All stages of larvae were fed on ground fish food (TetraMin® tropical flakes, Tetra®, Blacksburg, VA, USA) and adults were provided with 10% sucrose solution ad libitum.

2.3. Selection and Resistance Profiling

The five insecticide resistant strains were routinely selected every 3rd to 5th generation with 0.05% deltamethrin to preserve their resistant phenotype. Insecticide papers were purchased from the WHO facility at the University Sains Malaysia (USM), Penang, Malaysia and used a maximum of 6 times following the WHO procedure [24]. Selection was undertaken at the adult stage (2–5 days old) using the WHO susceptibility bioassay [24]. Exposure times varied between strains to ensure at least 50% survival (VK7 2014 2 h, Banfora M and Bakaridjan 2–3 h, Gaoua-ara 2–4 h and Tiefora 4–5 h). All adults from the generation to be selected were exposed, with results scored from at least 100 individuals. Following exposure, the mosquitoes were transferred to holding tubes and supplied with 10% sucrose solution and the initial knockdown effect was scored immediately post exposure. At 24 h post exposure, mortality rates were recorded. Bioassays and 24 h holding periods were conducted at 26 ± 2 °C and 80 ± 10% RH.

Table 1. Summary of the *Anopheles gambiae* s.l. mosquito strains used in the study.

Strain	Species	Origin	Source	Year Colony Established
Kisumu (susceptible strain)	*An. gambiae* (s.s.)	Kenya	MR4	1975
N'Gousso (susceptible strain)	*An. coluzzii*	Cameroon	CRID	2006
Moz (susceptible strain)	*An. arabiensis*	Chokwe, Southern Mozambique (24° 33′ 37″ S, 33° 1′ 20″ E)	Established in LSTM from field collections performed by JCM with assistance from National Institute of Health, Mozambique [23]	2009
VK7 2014	*An. coluzzii*	Houet Province, Burkina Faso Valley de Kou 7 (11°24′29″ N, 4°24′37″ W)	Established from larval collections performed by LSTM (JCM) and CNRFP (KHT)	2015
Banfora M	*An. coluzzii*	Comoé Province Burkina Faso Banfora district (Tiefora, Pont Maurice, Sikane and Djomale (10° 38′ 0″ N, 4° 33′ 0″ W) and Bakaridjan (10°24′26.34″ N, 4°33′44.78″ W) villages)	Established from larval collections performed by LSTM (JCM) and CNRFP (KHT)	2015
Bakaridjan	*An. gambiae* (s.s)	Comoé Province Burkina Faso Banfora district (Tiefora, Pont Maurice, Sikane and Djomale (10° 38′ 0″ N, 4° 33′ 0″ W) and Bakaridjan (10°24′26.34″ N, 4°33′44.78″ W) villages)	Established from larval collections performed by LSTM (JCM) and CNRFP (KHT)	2015
Tiefora	*An. coluzzii*	Comoé Province, Burkina Faso Banfora district (10° 37.447′ N, 4° 33.201′ W)	Established from larval collections performed by LSTM (JCM) and CNRFP (KHT)	2018
Gaoua-ara	*An. arabiensis*	Poni Province Burkina Faso Gaoua district (10.3231° N, 3.1679° W)	Established from larval collections performed by IRSS (ASH)	2018

Each strain was profiled annually against eight insecticides (except VK7 2014 which was profiled against six insecticides) representing the different insecticide classes currently used for mosquito control, to monitor the stability of their resistance phenotype; as described in [9] insecticides used were permethrin, deltamethrin, alpha-cypermethrin, DDT, dieldrin, bendiocarb, propoxur and fenitrothion. Results for VK7 2014 and Banfora M have been reported previously [9], but are included here for comparative purposes.

The intensity of resistance was evaluated in the different strains using papers treated with 5× and 10× the diagnostic dose of permethrin following the WHO procedure [24].

Figure 1. Map of Burkina Faso showing mosquito collection sites.

2.4. Synergist Bioassays

The impact of the synergist piperonyl butoxide (PBO) on pyrethroid induced mortality in each of the resistant strains was assessed in two separate experiments. Firstly, 2–5 day old female mosquitoes were pre-exposed to PBO papers impregnated with PBO (4%) followed by 1, 2, 3 or 4 h exposures to papers impregnated with permethrin (0.75%) according to the WHO protocol [24].

In the second experiment, mortality rates following sequential PBO then pyrethroid exposure were compared with simultaneous exposure to insecticide and synergist. Adult females from three strains were exposed to either (1) a pyrethroid only 1-h exposure; (2) a 1-h PBO pre-exposure followed by a 1-h pyrethroid exposure, or (3) a 1-h combination exposure (with PBO and either pyrethroid on the same paper). These experiments were performed separately using 0.75% permethrin papers and 0.05% deltamethrin papers.

In both experiments, solvent only paper (no AI) and a PBO control, where a 1-h PBO exposure was followed by 1-h blank exposure were included. Differences in mortality with and without PBO exposure were analysed for significance using Fisher's exact test.

2.5. Cone Bioassays

Mosquitoes were exposed to PermaNet® 3.0 LN (Vestergaard Frandsen SA, Denmark) a LLIN consisting of a top panel made of monofilament polyethylene (100 denier) fabric incorporating deltamethrin at 4 g/kg (approx. 180 mg/m^2) and piperonyl butoxide at 25 g/kg (approx. 1.1 g/m^2), plus side panels made of multifilament polyester (75 denier) fabric with a strengthened border treated with deltamethrin at 2.8 g/kg (approx. 118 mg/m^2) in WHO cone bioassays [25]. Following net airing of 2 weeks, pieces of netting (25 cm × 25 cm) were cut from the roof and side of the PermaNet 3.0 and cohorts of approximately 50 mosquitoes of each strain were exposed using the WHO standard protocol. Controls were exposed to insecticide free net in two replicates, each with 5 mosquitoes, one just before and one just after the treated exposures. Following exposure, the mosquitoes were aspirated into paper cups and supplied with 10% sucrose solution, and the initial knockdown effect was scored at 1 h and mortality was scored at 24 h post exposure.

2.6. Target Site Mutation Genotyping

Genomic DNA was collected within the first 5 months of colonisation of each strain and every subsequent 6–12 months thereafter. The DNA was extracted from 48 non-blood-fed females using a Qiagen blood and tissue DNA extraction kit (Qiagen, Germantown, MD, USA). Species ID was identified using the SINE PCR protocol [21].

Each strain was genotyped to identify the frequency of known target site resistance alleles (alleles 995F, 995S and 1570Y in the VGSC, the *ace-1*119S allele and the RDL alleles 296G and 296S) using Taqman™ assays [26–29]. The allelic variant 114T of the glutathione transferase *GSTe2* gene was also genotyped as previously [30].

2.7. RNAseq Transcriptomic Analysis

RNA was extracted from pools of 5, 3–5 day old presumed-mated adult females, snap frozen in the −80 °C at 10 am, using a PicoPure kit (Applied Biosystems Thermo Fisher, Waltham, MA, USA, after homogenisation with a motorised pestle. Quality and quantity of the RNA was analysed using an Agilent TapeStation (Santa Clara, CA, USA) and Nanodrop (Thermo Fisher) respectively, and three (Moz, Gaoua-ara, N'Gousso, Tiefora) or four (Banfora, VK72014, Kisumu, Bakaridjan) replicates from each strain sent for sequencing at Centre for Genomics, Liverpool, UK (RNA extractions for Banfora were performed as part of a separate study [31] but using the same methodology).

The resulting data was run through appropriate QC using FastQC and aligned to the latest *Anopheles gambiae* s.l. genome assembly PEST4 using Hisat2 with default parameters. The resulting bam file was sorted using samtools and the number of reads aligned to each gene extracted using featureCounts. Over 70% read assignment was seen for each replicate of each population with the majority showing >85%. Data from the *An. gambiae* s.s and *An. coluzzii* resistant populations were compared to the two susceptible populations (Kisumu and N'Gousso) using limma. First, a model matrix was defined to account for the populations and then contrasts were made to compare the resistant *An. gambiae* and *An. coluzzii* to both susceptible populations through the function makeContrasts using resistant—(N'Gousso + Kisumu)/2. Counts were then transformed to log2 counts per million reads (CPM), residuals calculated, and a smoothed curve fitted using the voom function which utilises normalisation factors calculated using calcNormFactors. lmFit was used to fit a linear model for each gene and eBayes used to smooth the standard errors. The function topTable was then used to retrieve results and written out to file; significance was taken as adjusted p value ≤ 0.05. In the case of the single *An. arabiensis* population, the contrast matrix was simply a resistant vs. susceptible design. In each instance the filterByExpr function from the EdgeR package was used to remove genes with low read number. Enrichment analysis was performed using the built-in GO term enrichment analysis on VectorBase with a Benjamini significance cut-off of ≤ 0.05. Revigo was then used to remove redundant GO terms allowing more appropriate visualisations; default parameters were used with a 0.5 selection. A custom table was also used with hypergeometric tests with fdr cut-off of ≤ 0.05 to integrate KEGG, Reactome and a priori genes of interest into the enrichment analysis (https://github.com/VictoriaIngham/BurkinsStrains) (accessed on 9 December 2021). All RNAseq data is deposited in SRA under accession PRJNA780362 and PRJNA750256.

2.8. Metabolic Resistance—Detox Gene Expression Levels

One to four µg of RNA, extracted from three pools of 5, 3–5-day-old female as described above, was reverse transcribed using Oligo dT (Invitrogen, Warrington, UK) and Superscript III (Invitrogen). The resulting cDNA was diluted to 4 ng/µL and used as a template in the subsequent PCR reactions. Primers and probes as described by Maviridis et al. [32] were ordered from Integrated DNA Technologies (Leuven, Belgium), with Cy5 replacing Atto647N. Primers and probes were diluted to 10 µM for use in a 10 µL final reaction. Four multiplex reactions were carried out on each cDNA set in technical triplicate, as follows: (i) CYP6P4, CYP6Z1 and RPS7; (ii) CYP4G16 and CYP9K1; (iii) CYP6M2 and CYP6P1;

(iv) CYP6P3 and GSTE2. PrimeTime Gene Expression Master Mix (Integrated DNA Technologies) was used to set up each reaction following the manufacturer's instructions. Each reaction was carried out on a MxPro 3005P qPCR System (Agilent) with the following thermocycling conditions: 3 min at 95 °C followed by 40 cycles of 15 s at 95 °C; 1 min at 60 °C. Cycle threshold (Cq) values were exported and analysed using the ΔΔct methodology [33], using RPS7 as an endogenous control. Gaoua-ara were normalised against the susceptible Moz strain of *An. arabiensis*, and Bakaridjan, Banfora M, Tiefora and VK7 2014 were compared to both N'Gousso and Kisumu. A homogeneity of variance test was used to determine if data were normally distributed. Δct values were transformed to normalise (where applicable) and an ANOVA test, followed by Dunnett's test was performed. Where transformations did not normalise the data, a Dunn test was performed.

3. Results

3.1. Discriminating Dose Assays

Bakaridjan, Gaoua-ara, Banfora M, Tiefora and VK7 2014 are all resistant to pyrethroids and DDT according to WHO definitions [24] (Figure 2). Gaoua-ara and Tiefora are also resistant to the organochlorine dieldrin. Bakaridjan, Gaoua-ara and Tiefora are resistant to the carbamates propoxur and bendiocarb with Banfora M resistant to bendiocarb only. None of the five strains are resistant to the organophosphate fenitrothion. Kisumu and Moz are susceptible to all the insecticides tested and results have been reported previously [9]. N'Gousso showed less than 90 % mortality after exposure to propoxur (87%), DDT (61%) and dieldrin (39%) but was susceptible to other insecticides tested.

The results of profiling the five resistant strains against 5 and 10× diagnostic dose (DD) of permethrin are shown in Figure 3. All 5 strains survived exposure to 5× DD (mortality ranged from 14% to 71%). Four of the strains also showed less than 90% mortality after exposure to 10 × permethrin papers (and would be described by WHO as having high intensity resistance) whereas Gaoua-ara with 55% mortality with 5× papers, 98% mortality with 10× is defined by WHO as moderate to high intensity resistance.

3.2. Impact of PBO on Pyrethroid Mortality

All strains showed significant synergism when pre-exposed to PBO followed by a 4-h exposure to permethrin but synergism was not consistently observed with shorter pyrethroid exposures and PBO pre-exposure did not fully restore susceptibility to permethrin in any of the strains (Figure 4; full mortality results and synergism ratios are available in Supplementary Table S1). The highest synergist ratios were seen for Banfora where significant synergism was observed at all permethrin exposures greater than 2 h and PBO:permethrin synergism ratios ranged from 7:1 (2 h) to 54:1 (3 h). Negative controls (both control papers only and PBO followed by control papers) gave <4% mortality in all assays.

In a separate set of experiments the effect of sequential versus simultaneous exposure to PBO and pyrethroids was compared (Supplementary Figure S1) with pyrethroid exposure duration constant at one hour. PBO did not synergise permethrin in these experiments but the efficacy of deltamethrin was significantly improved in all three strains with both PBO exposure methods ($p < 0.0001$ in all cases). Simultaneous exposure to PBO and pyrethroids results in increased mortalities compared to PBO pre-exposure for all three strains but this was only significant ($p < 0.05$) in Bakaridjan for both insecticides and in Banfora with deltamethrin. Full mortality results and synergism ratios are available in additional Supplementary Table S2.

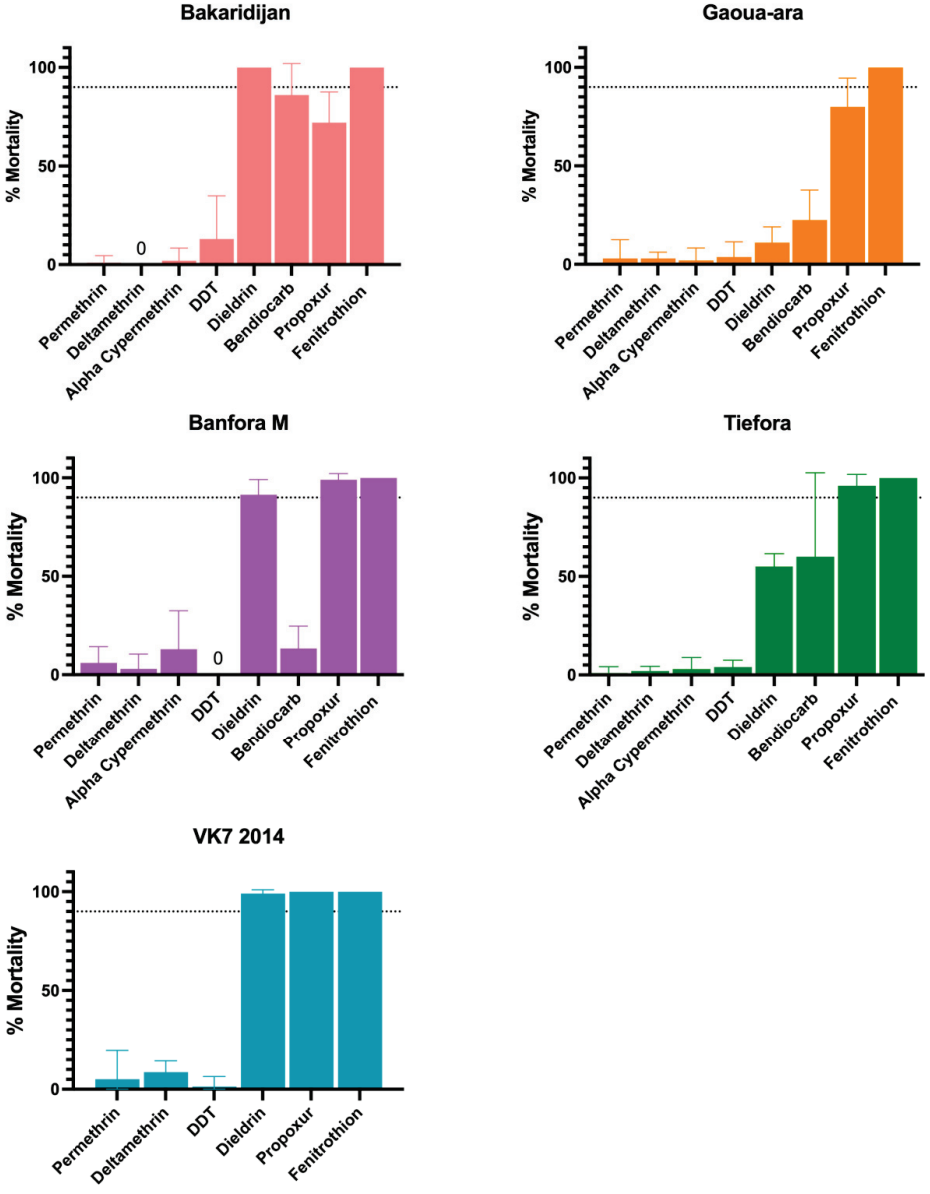

Figure 2. Mosquito mortality following exposure to insecticide papers in discriminating dose assays Mortality rates (%) 24 h after exposure for 5 strains of Anopheles mosquito (results shown from assays performed in 2019). Minimum sample size n = 80. Error bars represent 95% confidence intervals. Dotted line represents the WHO 90 % mortality resistance threshold.

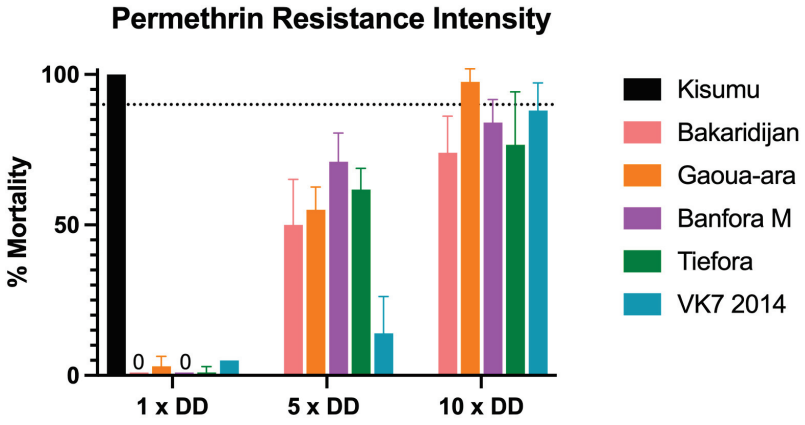

Figure 3. Mosquito mortality following exposure to permethrin papers in WHO resistance intensity assays. Mortality rates (%) 24 h after exposure for 5 strains of Anopheles mosquito. Minimum sample size n = 80. Error bars represent 95% confidence intervals. Dotted line represents the WHO 90% mortality resistance threshold. DD: Diagnostic dose.

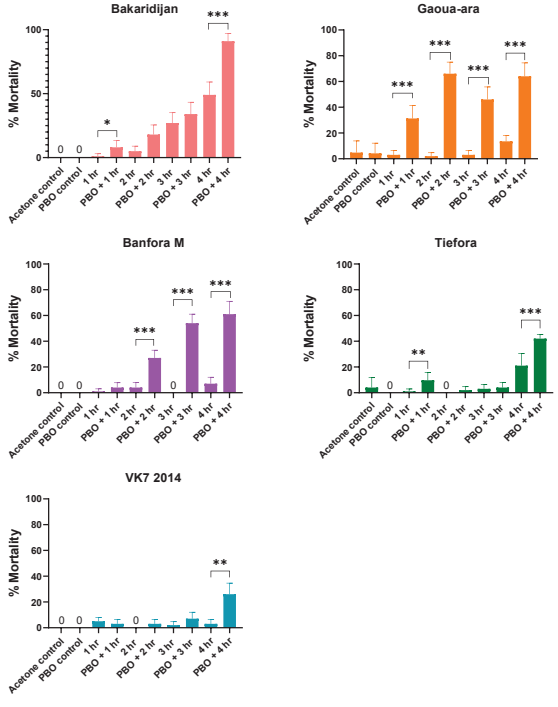

Figure 4. Mortality following exposure to permethrin with or without the synergist PBO. Mortality rates % 24 h after exposure. Minimum sample size n = 80. Error bars represent 95% confidence intervals. Statistical differences between permethrin only and PBO + permethrin for each paired combination indicated as * p <0.05, ** p < 0.001, *** p < 0.001 Fisher's Exact test.

3.3. Cone Bioassays

Exposure to the side of the PermaNet 3.0 net in a cone bioassay consistently resulted in <10% mortality for all 5 strains but exposure to the roof (containing PBO) resulted in >98% mortality (Figure 5).

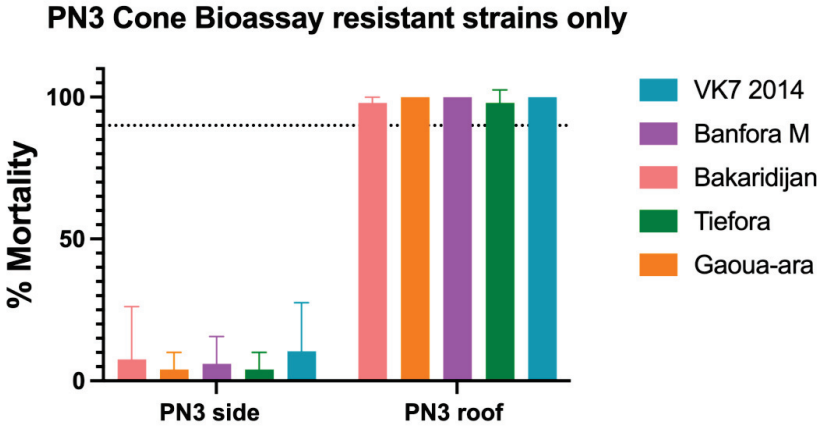

Figure 5. Mortality following exposure to Permanent 3.0 LLINs (PN3) in cone bioassays. Mortality rates % 24 h after exposure. Minimum sample size n = 50. Error bars represent 95% confidence intervals.

3.4. Target Site Mutation Genotyping

All the strains were screened for five target site mutations and one mutation in a detoxification gene (Figure 6). The 995F kdr allele was fixed in Bakaridjan and VK72014, but was present at quite low frequencies in Tiefora (allele frequency 0.06) and Banfora M (allele frequency 0.38). The *An. arabiensis* Gaoua-ara strain contained both 995F and 995S with allele frequencies of 0.49 and 0.45, respectively. The 1570Y kdr allele was detected in Bakaridjan, VK7 2014, Banfora M and Tiefora with allele frequencies of 0.04, 0.35, 0.48 and 0.04, respectively. The ace-1 mutation was absent from all strains except a very low frequency in the Tiefora strain. The RDL 296S allele was detected in Gaoua-ara, Banfora M, VK7 2014, and Tiefora with allele frequencies of 0.65, 0.22, 0.03 and 0.26, respectively; only the wildtype form of A296 was found in the *An. gambiae* Bakaridjan strain. The GSTE2 114T detox gene modification was found in Banfora M, VK7 2014 and Tiefora with allele frequencies of 0.66, 0.77 and 0.46, respectively.

3.5. RNAseq Analysis

RNAseq analysis was carried out on a minimum of three biological replicates from the five resistant strains and the three laboratory susceptible colonies, Kisumu, N'Goussu and Moz. The correlation matrix shows high degrees of similarity between the two *An. arabiensis* populations, Gaoua-ara and Moz but no clear segregation according to species for the *An. gambiae* and *An. coluzzii* strains (Supplementary Figure S2). Hence, for all further analysis of differential expression between resistant and susceptible strains, Gaoua-ara was compared to Moz alone whereas the three *An. coluzzii* (Tiefora, VK72014, Banfora) and one *An. gambiae* s.s.(Bakaridjan) resistant strains were compared to the average values from the two susceptible strains of *An. coluzzii* (N'Gousso) and *An. gambiae* (Kisumu).

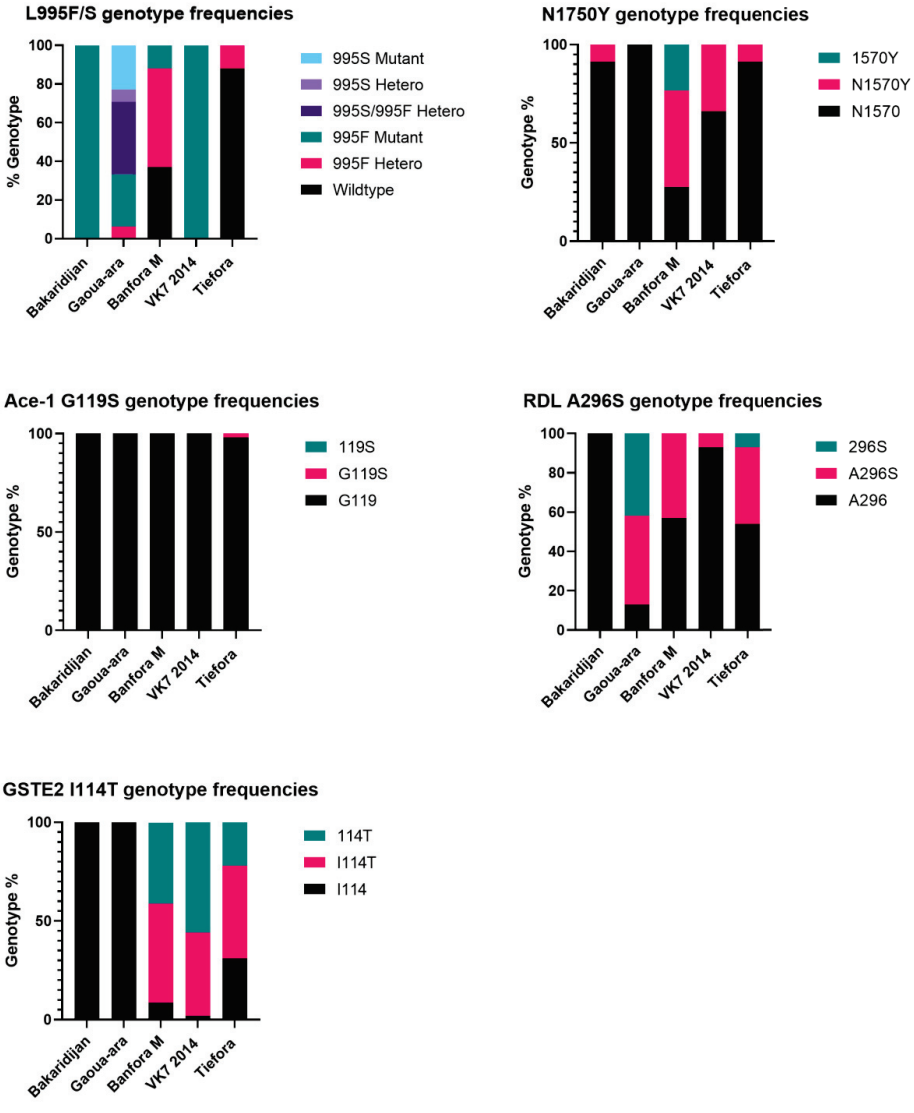

Figure 6. Frequency of point mutations associated with resistance. Data reported from samples genotyped in 2019. 995L, 1575N, 119G, 269A and 114I indicate the wildtype genotype (black bars); 995F, 995S, 1570Y, 119S, 296S and 114T indicate resistant genotype (green or purple bars). Heterozygote genotypes are shown with pink bars.

3.6. Similarities between Strains

The total number of genes differentially expressed across all the resistant compared to susceptible strains is shown in Supplementary Table S3. A total of 81 transcripts were up regulated in resistant versus susceptible strains with 73 down regulated. The upregulated transcripts show no enrichment but two P450s known to bind and/or metabolise pyrethroids (CYP6P3 and CYP6Z2) are amongst the most highly upregulated genes and two glucoronosyl transfearses (UGT302H2 and UGT306A2) are also elevated in all strains.

Down regulated transcripts are strongly enriched for RNA processing ($p = 1.25 \times 10^{-4}$) and do not contain genes previously associated with pyrethroid resistance.

GO term enrichment was explored for each individual resistant population. Whilst no GO terms were enriched across all five resistant populations, a number of similarities were seen across the four resistant *An. gambiae* and *An. coluzzii* colonies (Supplementary Figure S3). GO terms significant in up-regulated genes across each population include oxidoreductase activity, typically seen in resistant colonies [34,35] and related to cytochrome p450 activity, and terms related to neuronal signalling, potentially indicating changes in signalling and neurotransmitter activity are associated with resistance to these neurotoxic insecticides. Additionally, terms related to ATPase activity and GPCR signalling, both previously linked to insecticide resistance [36,37] are seen. There are similarities in GO enrichments in the down-regulated subset of genes, with terms related to transcription factor activity, translational regulation, regulation of dephosphorylation and phosphatase complexes, all repressed (Supplementary Figure S4).

3.7. Differences between Strains

The RNAseq data was then interrogated to identify both pathways and *a priori* candidate genes enriched in the up or down regulated genes in each resistant strain with *An. gambiae* and *An. coluzzii* compared to two susceptible controls. Analysis at the individual gene level revealed key differences between the strains. For example, 23 P450s are differentially expressed in one or more strains; as mentioned above, CYP6P3 and CYP6Z2 are up-regulated in all resistant strains but other known pyrethroid metabolisers including CYP6M2, CYP6P2, P4 and P5 and CYP9J5 and 9K1 [38,39] are also up-regulated in two or more strains (Figure 7). This analysis also identifies a number of additional P450s that are highly up-regulated in multiple strains but have not yet been functionally characterised (e.g., CYP4H genes) which merit further study.

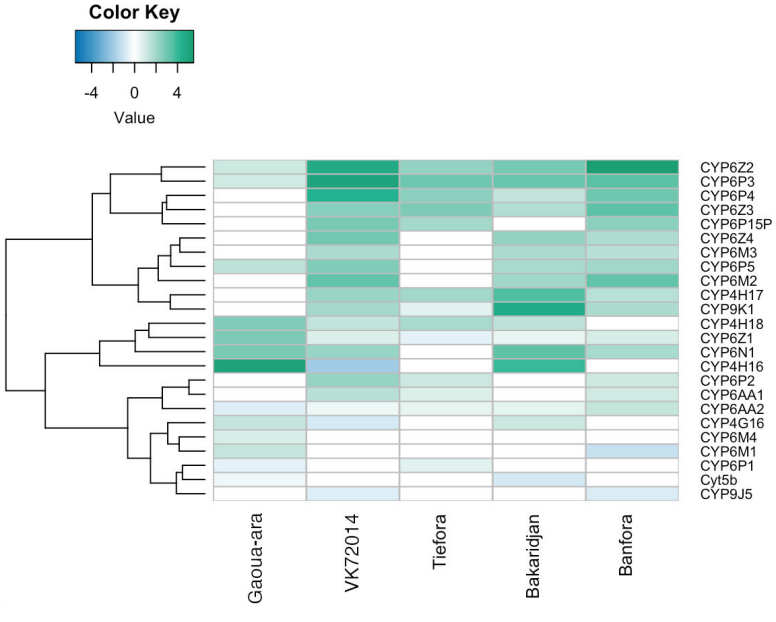

Figure 7. Heatmap showing cytochrome P450 genes that are significantly differentially expressed between the pyrethroid resistant and susceptible strains.

Recently, a number of genes with putative roles in sequestering pyrethroids were found to be over expressed in pyrethroid resistant populations from West Africa [40].

RNAseq data from the *An. gambiae* complex in Burkina Faso is supportive of a putative role of hexamerins in pyrethroid resistance in *An. arabiensis* and the VK7 strain of *An. coluzzii* (as shown previously [37]) (Figure 8). Suppression of the hexamerin AGAP001659 (highly upregulated in Gaoua-ara in this study) was previously associated with a reduction in pyrethroid resistance [37]. In addition, several alpha- cyrstallins were up-regulated in one or more of the pyrethroid resistance populations, with this gene family particularly enriched in the Banfora strain of *An. coluzzii* in agreement with earlier qPCR data [40]. Suppression of the alpha-cyrstallin AGAP007159, which is upregulated in multiple Burkina populations, has also been shown to result in a reduction in the resistance phenotype in VK7 2014.

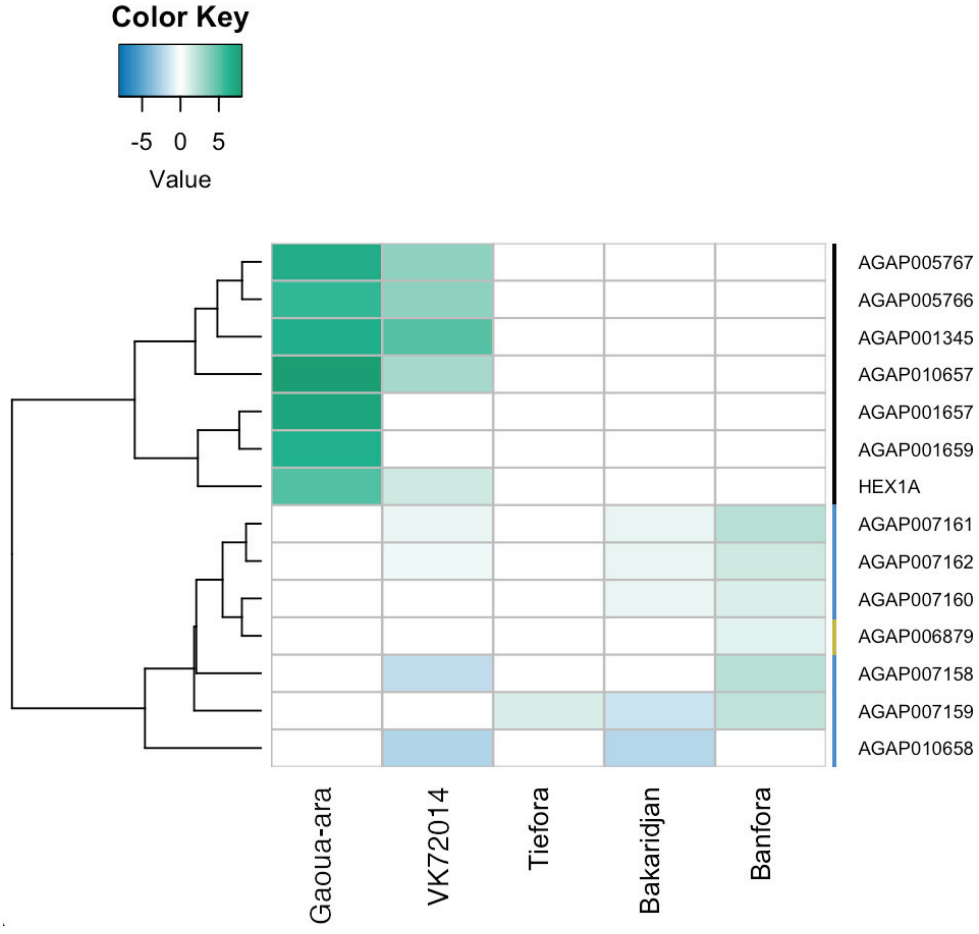

Figure 8. Heatmap showing differential expression of genes in families putatively associated with insecticide sequestration between the pyrethroid resistant and susceptible strains.

Finally, we looked at expression of genes recently implicated in the cuticular hydrocarbon (CHC) synthetic pathway. This gene list was derived from transcripts encoding the six gene families (propionyl co A synthases, fatty acid synthases, elongases, desaturases, reductases and P450 decarbonylases) that are enriched in the sub epidermal oenocyte cells responsible for CHC production [41]. Several genes in this pathway were up-regulated in the Banfora, Bakaridjan and Gaoua-ara strains but, surprisingly, down-regulated in two of

the strains, Tiefora and VK7 2014 (Supplementary Figure S5). To date only two genes in this putative pathway have been functionally validated, CYP4G16 [42] and the fatty acid synthase FAS1899 [41]; both of these genes are upregulated in the pyrethroid resistant *An. arabiensis* strain (fold changes of 5.2 and 2- fold respectively) suggesting that cuticular resistance may be a particularly important resistance phenotype in this population.

3.8. Evaluation of a Multiplex Gene Expression Assay for Metabolic Resistance

RNAseq analysis provided a list of putative genes and pathways potentially contributing to the pyrethroid resistance phenotype in the different strains. However, simpler robust assays of gene expression are needed to further investigate the association between gene expression and resistance phenotype. To this end, the Taqman multiplex assay [32] was used to quantify relative expression of a subset of 8 insecticide detoxification genes in each of the resistant strains compared to their susceptible counterparts (to facilitate correlations with RNAseq data, expression levels from the *An. gambiae* and *An. coluzzii* resistant strains were compared to the average expression of the equivalent transcripts in the *An. gambiae* and *An. coluzzii* susceptible strains). The data generated in this study agreed well with previous Taqman multiplex P450 expression data for VK7, with the exception of CYP9K1 (where significant up-regulation was not detected in earlier generations). P450 levels in Banfora appear more variable between generations, consistent with recent findings that the resistance phenotype is less stable in this population than in other laboratory colonies [28]. Within the current study, there is generally good agreement between the qPCR (Supplementary Figure S6 and Supplementary Table S4) and RNAseq data, with the exception of CYP6P3 and CYP6Z1 (Table 2).

Table 2. Summary of correlation between results of detoxification multiplex qPCR and RNAseq data.

	An. coluzzii			*An. gambaie*	*An. arabiensis*
	VK72014	**Banfora**	**Tiefora**	**Bakaridjan**	**Gaoua-ara**
CYP4G16					
CYP6M2					
CYP6P1					
CYP6P3					
CYP6P4					
CYP6Z1					
CYP9K1					
GSTE2					

Genes up-regulated in both qPCR and RNAseq data set
Genes up-regulated in qPCR data set only
Genes up-regulated in RNAseq data set only

4. Discussion

This study provides a detailed description of the extent and causes of pyrethroid resistance in three new colonies of *An. gambiae* s.l. from Burkina Faso and provides further information on the genetic basis of pyrethroid resistance in two colonies originating from the same region and described previously [9].

The high levels of pyrethroid resistance present in all five resistant strains, from three different species, reinforces the view that Burkina Faso is a hotspot of resistance [3,43–45]. All colonies were maintained under deltamethrin selection and data from WHO intensity assays show little difference in resistance levels between the strains. Although technically the *An. arabiensis* colony is defined as moderately resistant whereas the four *An. coluzzii* and *An. gambiae* strains meet the definition of high resistance, when time of exposure, rather than concentration of insecticide, was the variable, the *An. gambiae* s.s strain was the least resistant of the strains. Bioassays conducted in Burkina Faso in 2010 found that both *An. gambiae* and *An. coluzzii* were significantly more likely to survive permethrin exposure than *An. arabiensis* [46]; these species differences now seem to have been largely eroded, at least in the Burkina Faso populations assayed in this study. Several of the

strains also showed resistance to other insecticide classes including carbamates and the cyclodiene, dieldrin. These insecticides are not used for mosquito control in this region and hence the observed resistance may be indicative of agricultural exposure selecting for resistance [3] (or alternatively may be explained by cross resistance between insecticide classes, see below). Insecticides from additional classes including the neonicotinoids and pyrrole, are now being incorporated into vector control products such as indoor residual sprays and ITNs and work is ongoing to assess the susceptibility of these laboratory colonies to these active ingredients. Encouragingly, all strains appear susceptible to the pyrrole chlorfenapyr, used in the ITN IG2® (BASF, Germany) that is being deployed in pilot schemes in Burkina Faso [47].

Pre-exposure to the synergist PBO, did increase permethrin induced mortality but could not fully restore susceptibility in any strain. Simultaneous exposure to PBO and pyrethroids typically resulted in higher mortalities than observed after sequential exposure, perhaps indicating that PBO acts as an adjuvant, as well as an inhibitor of P450s, as has been proposed previously [48] but mortality rates were still well below 100% mortality. However, when mosquitoes from all five strains were exposed to a formulated product containing PBO (the roof of a Permanent 3.0 ITN) 100% mortality was observed after just a 3 min exposure. This highlights the challenges of interpreting results from different bioassays and extrapolating to field effectiveness. High mortalities after exposure to ITNs containing PBO has been observed previously in cone bioassays on *An. coluzzii* from this region and experimental hut studies conducted the same year (2014) showed that PBO ITNs caused higher mosquito mortalities than standard pyrethroid only ITNs [49]. However, rising levels of pyrethroid resistance in the region, appear to be undermining the effectiveness of PBO nets (WMG, N'FS, unpublished data).

As expected, mutations in the VGSC gene, the target site of pyrethroids, were found in all strains, but there was a surprising variation in the frequency of the 'typical' kdr haplotypes, 995F and 995S. The 995S allele was only found in *An. arabiensis* and was found in approximately equal frequency to the 995F allele, with the most prevalent genotype being 995F/995S heterozygotes. Similar heterozygotes have been detected in Cameroon and Gabon, with some evidence of a fitness advantage [13]. The 995S allele was first reported in *An. arabiensis* in Burkina Faso in 2008 [46] and the reasons it remains confined to this member of the complex are unknown. The *An. gambiae* Bakaridjan strain and *An. coluzzii* VK7 2014 are both fixed for the 995F allele but this SNP was found at very low frequencies in the other two resistant *An. coluzzii* strains. Subsequent further investigations have detected an alternative VGSC haplotype in pyrethroid resistant *An. coluzzii* from Burkina Faso, consisting of a double mutation at codons 402 and 1527 [14] and have shown that the Banfora M and Tiefora laboratory colonies contain high frequencies of this 402L:1527T haplotype, which is mutually exclusive with the 995F haplotype. The functional significance of the two alternative VGSC resistance haplotypes is the subject of ongoing investigations, comparing the resistance phenotype and fitness costs, and genotyping resistant mosquitoes from neighbouring regions, to try and establish why there is an apparent evolutionary shift away from 995F to alternative amino acid substitutions in these *An coluzzii* populations. In the context of the current study, it is interesting that the 402L:1527T haplotype is only present in one species of the colonies of *An. gambiae* s.l. that were established from the same larval collections in the same breeding sites (Bakaridjan and Banfora M). Introgression of kdr alleles between members of the *An. gambiae* complex has occurred on multiple occasions [50] and longitudinal monitoring of the frequency of these alternative haplotypes in the Cascades region of Burkina Faso may provide an opportunity to monitor any further genetic exchange in this genomic region.

The three new strains described in the current study all contain some level of carbamate resistance, but the target site allele Ace-1 is absent in two of the strains and found at very low frequencies in the third (Tiefora). The persistence of carbamate resistance in these strains for multiple generations in the insectary, in the absence of carbamate selection, together with the absence of target site resistance, point to possible cross resistance between

pyrethroids and carbamates. CYP6P3, which is elevated in all of the resistant strains, has been shown to metabolise a wide range of insecticides from different classes, including the carbamate bendiocarb [39,51].

The 'resistance to dieldrin' Rdl allele 296S is found at frequencies exceeding 20% in the three newly described strains and its frequency broadly correlates with the prevalence of dieldrin resistance in these strains, with Gaoua-ara (Rdl frequency 0.65) the most resistant to dieldrin. The point mutation GSTE2–114T, which results in an enhanced version of the detox gene Gste2 known to metabolise DDT [30], was found in the three *An. coluzzii* strains at relatively high frequencies (above 0.46 in all cases). All of these strains are highly resistant to DDT; however, the contribution of the GSTE2-114T allele to DDT resistance is difficult to assess in these strains given the presence of target site resistance and the finding that expression levels of GSTE2 are elevated in these resistant strains

RNAseq was used to identify additional resistance mechanisms potentially contributing to the intense pyrethroid resistance phenotype in these strains. The up-regulation of several P450s, together with the partial synergism conferred by PBO, confirmed the importance of this mechanism with many of the known pyrethroid metabolisers up-regulated in multiple strains and the three subfamilies (6P, 6M and 6Z) most widely associated with pyrethroid resistance amongst [39] the most up-regulated, particularly in the *An. coluzzii* strains. Interestingly, in the *An. arabiensis* and *An. gambiae* populations, some of the strongest candidates, based on expression levels alone, are found in other subfamilies of P450s, notably the CYP4H family for *An. arabiensis* which has been implicated in pyrethroid resistance in previous microarray studies [23,52,53] but has not, to our knowledge, been functionally characterised.

In addition, genes thought to play a part in the synthesis and deposition of hydrocarbons on the mosquito cuticle [41] were up-regulated in some strains. Elevated levels of cuticular hydrocarbons have been associated with pyrethroid resistance in *An. coluzzii* mosquitoes from Valle du Kou [10,54] in Burkina Faso and evidence of an association between epicuticle thickness and insecticide resistance has been reported in several additional Anopheles populations [11,55]. As this resistance mechanism may confer cross resistance to a wide range of contact insecticides, it is important that insecticide screening pipelines incorporate strains with thickened cuticles. However, our own observations indicate that this mechanism may be less stable in laboratory colonies than other resistance mechanisms, perhaps indicative of a high fitness cost which is balanced by other phenotypic advantages, such as ability to withstand desiccation [56], or mating advantage [10].

Further putative resistance mechanisms are indicated by examination of the RNAseq but have not been functionally validated. For example, two odorant binding proteins (AGAP000278 and AGAP012867) are up-regulated in all of the pyrethroid resistant populations from Burkina Faso. The chemosensory protein SAP2, expressed in mosquito legs and antennae, has already been shown to play a key role in pyrethroid resistance in *An. gambiae s.l* from Burkina Faso but [12], whilst OBPs have been associated with resistance in other studies [57,58], a direct role for this family in pyrethroid resistance remains to be demonstrated. Other gene families putatively involved in insecticide binding (and maybe sequestration) were elevated in multiple Burkina populations, most notably the hexamerins, found in the mosquito haemeolymph where they act as storage and transport proteins, which are highly enriched in the *An. arabiensis* resistant strain. The absence of DNA markers for these putative resistance mechanisms makes it difficult to evaluate their individual contributions to the phenotype but temporary loss of function via RNAi has been successfully used in the past to demonstrate a link between individual genes within putative insecticide binding protein families and resistance [40]. In vitro studies on recombinant proteins are also needed, both to confirm their role in pyrethroid binding, but importantly also to assess the ability to bind other insecticide classes.

5. Conclusions

This study demonstrates that different species within a species complex, collected from the same geographical area (including two originating from the same larval collections) and hence presumably under similar selection pressures, can evolve multiple, different resistance mechanisms. This may be indicative of the exceptionally strong selection pressure exerted on Anopheles mosquitoes in this major agricultural region in Burkina Faso but it presents a major challenge for existing and new insecticide based control tools. As the strains have been maintained under selection pressure in the laboratory, the fitness costs of alternative mechanisms, and hence their stability under natural settings, are unknown but nevertheless the strains represent a valuable biological resource for the screening of new insecticides for potential resistance liabilities. From an evolutionary perspective, genomic sequencing of these strains, coupled with further sampling of sympatric members of the species complex in the region, provides an opportunity to investigate the role of introgression versus de novo mutation, in the evolution of resistance, and in assessing the response to the introduction of ITNs with new classes of insecticides.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects13030247/s1, Supplementary Table S1. Complete mortality data for standard PBO bioassays plus PBO: perm synergism ratios for five resistant strains. Mean mortality rates (%) across three reps 24 h after exposure are given, minimum sample size 75. Supplementary Table S2. Complete mortality data for simultaneous and sequential exposures of PBO with either permethrin and deltamethrin plus PBO synergism rations for five resistant strains. Mean mortality rates (%) across three reps 24 h after exposure are given, minimum sample size 75. Supplementary Table S3. The total number of genes differentially expressed across resistant compared to susceptible strains. Supplementary Table S4. qPCR data showing expression levels of the panel of detoxification genes in the five resistant strains. Mean fold changes from 3 biological replicates and 3 technical replicates, relative to susceptible strain(s), SD = standard deviation. Data have been normalised against expression in the susceptible strain as described in the methods. Supplementary Figure S1: PBO synergism results for three resistant anopheline strains with simultaneous and sequential exposures to PBO and permethrin (Perm) or deltamethrin (Delta). Mortality rates % (24) hours after exposure. Minimal sample size n = 80. Error bars represent 95% binomial confidence intervals. Statistical differences between insecticide only and PBO + insecticide are indicated as * $p < 0.05$, or ns- not significant. Supplementary Figure S2: RNA Correlation matrix for five resistant strains and three susceptible strains. Red represents a strong correlation and blue represents a disassociation. VK7 = VK7 2014, Bak = Bakaridjan, T = Tiefora, K = Kisumu, B = Banfora M, NG = N'Gousso, M = Moz, G= Gaoua-ara. Supplementary Figure S3: GO terms enrichment up regulation for five resistant strains. Supplementary Figure S4: GO terms enrichment down regulation for five resistant strains. Supplementary Figure S5: Expression of genes in the cuticular hydrocarbon production pathway in five resistant strains. Blue represents genes down regulated and green represents genes upregulated. Reduct = reductase, Elong = elongase, Desat = desaturase, FAS = fatty acid synthase. Supplementary Figure S6: qPCR P450 expression in five resistant strains. Error bars represent standard deviations, statistically significant differences in expression level relative to susceptible strains are indicated as *$p < 0.05$, **$p < 0.01$, *** $p \leq 0.001$ ANOVA test followed by Dunnett's or Dunn test.

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Crystallography of Contemporary Contact Insecticides

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Simple Summary: The efficacy of crystalline contact insecticides is dependent foremost on the uptake of insecticide molecules by insect tarsi contacting crystal surfaces. Insecticide molecules, however, may organize in more than one way in the crystalline state, resulting in more than one crystalline form (also known as *polymorph*). We recently discovered that the lethality of contact insecticides increases with decreasing thermodynamic stability of the crystalline forms; the most stable crystalline form is invariably the least lethal/slowest acting. Polymorphism in contact insecticides, and its importance to efficacy, was largely unknown to the vector control community. It is argued that the crystallographic characterization of contact insecticide solids should be systematic to identify more active solid forms. Herein, we report seven new crystal structures, mostly pyrethroid insecticides recommended by the WHO for indoor residual spraying, as well as a new form of a neonicotinoid insecticide. These results further highlight polymorphism in contact insecticides and the importance of solid-state chemistry in the search for more active crystal forms.

Abstract: The active forms of contact insecticides used for combatting mosquito-borne infectious diseases are typically crystalline solids. Numerous molecular crystals are polymorphic, crystallizing in several solid forms characterized by different physicochemical properties, including bioavailability. Our laboratory recently found that the activity of crystalline contact insecticides is inversely dependent on the thermodynamic stability of their polymorphs, suggesting that efficacy can be enhanced by the manipulation of the solid-state structure. This paper argues that crystallography should be central to the development of contact insecticides, particularly because their efficacy continues to be compromised by insecticide resistance, especially among *Anopheles* mosquito populations that spread malaria. Although insecticidal compounds with new modes of action have been introduced to overcome resistance, new insecticides are expensive to develop and implement. The repurposing of existing chemical agents in metastable, more active crystalline forms provides an inexpensive and efficient method for 'evergreening' compounds whose risks are already well-established. We report herein seven new single-crystal structures of insecticides used for controlling infectious disease vectors. The structures reported herein include pyrethroid insecticides recommended by the WHO for indoor residual spraying (IRS)-bifenthrin, β-cyfluthrin, etofenprox, α-cypermethrin, and λ-cyhalothrin as well as the neonicotinoid insecticide thiacloprid.

Keywords: deltamethrin; imidacloprid; bifenthrin; β-cyfluthrin; etofenprox; α-cypermethrin; λ-cyhalothrin; thiacloprid; malaria; mosquitoes

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1. Introduction

Contact insecticides are often crystalline. These ingredients function when insect tarsi touch particle surfaces, leading the fight against malaria and other vector-borne diseases. Whilst much effort has been expended in the development of new compounds with improved efficacy, little attention has been paid to the solid-state structure of crystals that the insects encounter. Recent reports from our laboratory have revealed that the insecticidal activity of a particular contact poison depends on the crystal structures and

associated free energies of its solid forms, also known as polymorphs, which often are accessible under ambient conditions [1–5]. Some crystal forms of a given contact insecticide can knock down mosquitoes twelve times faster than the commercially available form [1,2]

The efficacy of insecticides is diminished by the development of resistance, which threatens the substantial progress made against malaria in this century [6–9]. The rapid uptake of an insecticide by insect tarsi upon contact with crystal surfaces is essential for overwhelming insecticide resistance, a consequence of various detoxification reactions [10,11]. If the rate of insecticide uptake can be increased, the toxicant may overwhelm resistance mechanisms. Whilst new insecticides, repellents, and anti-malarial compounds have been introduced in recent years, the introduction of new chemical agents in the field requires sizeable investments of labor and capital [12–15]. Consequently, the repurposing of existing chemical compounds through manipulation of their crystal structure can be faster, less expensive, and less risky because new compositions of matter are obviated [16].

Polymorphism, a common property of molecular solids [17], is the existence of two or more solid crystalline phases of the same compound. Weak intermolecular interactions and associated shallow potential energy hypersurfaces readily lead to solid forms with a different molecular organization in the solid state, accompanied by distinct chemical and physical properties among the different forms. The presentation of molecules at the crystal surfaces will differ among a family of polymorphs, leading to differences in the chemical potential of molecules at the surface. This is expected for each symmetry-independent facet of a given polymorph as well. The ease of cuticular extraction of insecticide molecules from crystal surfaces would be expected to increase with the increasing chemical potential of the crystal surfaces.

We demonstrated previously that metastable forms of insecticides such as DDT (dichlorodiphenyltrichloroethane), lindane, and fluorinated DDT congeners have greater activity than their most thermodynamically stable polymorphs [3–5]. A second DDT polymorph (Form II), first identified by McCrone [18], was characterized and found to be more active than Form I against *Drosophila melanogaster* [3].The inverse correlation between lethality and thermodynamic stability of polymorphs was demonstrated further by two newly characterized polymorphs of lindane, Forms II and III. Knockdown measurements for lindane Forms I, II, and III against *Drosophila melanogaster* revealed that the least stable polymorph kills twice as fast as the commercial Form I [4]. We also discovered a new crystalline form of the difluoro congener of DDT, DFDT (1,1′-(2,2,2-trichloroethane-1,1-diyl)bis(4-fluorobenzene)), as well as its amorphous form. The amorphous form (the least thermodynamically stable solid) was approximately three times faster acting than the thermodynamically stable form (Form I) towards *Anopheles quadrimaculatus* [5]. Moreover, chiral MFDT (1,1,1-trichloro-2,2-(4-chlorophenyl)-(4-fluorophenyl)ethane), a monofluorinated congener of DDT, also exhibited the inverse correlation between crystal thermodynamic stability and insecticidal activity.

We also observed identical trends for newly discovered polymorphs of deltamethrin (DM) and imidacloprid (IMI) [1,2], among the most widely used insecticides today. A second DM polymorph, denoted Form II after structural characterization by our laboratory [1], was found to be nine and twelve times faster acting than Form I against *Aedes aegypti* and *Anopheles quadrimaculatus* mosquitoes, respectively [1]. The two polymorphs not only differ with respect to the molecular arrangement in the solid state and the molecular presentations at their crystal surfaces, but they also differ with respect to the conformation of the DM molecules in the crystal lattice (Figure 1). Subsequently, we discovered new polymorphs of imidacloprid with different molecular conformations [2], the least stable polymorph exhibiting nine times greater activity against these mosquitoes than the commercial thermodynamically stable form. Importantly, these metastable forms were found to be stable against transformation to the thermodynamically stable form for at least six months, meeting World Health Organization guidelines for practical use in the field.

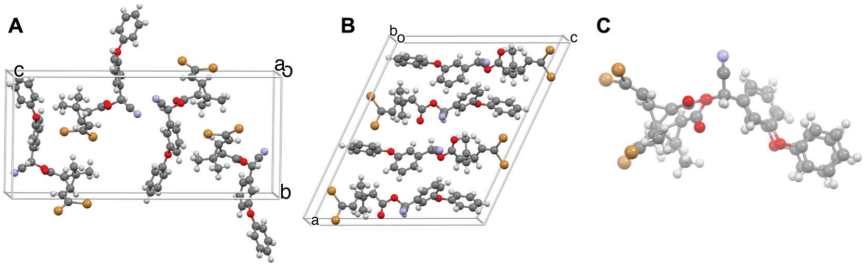

Figure 1. (**A**) Single-crystal structure of DM Form I. (**B**) Single-crystal structure of DM Form II. (**C**) The conformations of DM in Forms I and II overlaid, illustrating distinct molecular conformations in the polymorphs. The crystal structure of Form II was reported for the first time by our laboratory [1]. The crystal structure of DM Form I, redetermined by our laboratory is identical to that previously reported [19].

The role of polymorphism in contact insecticide formulations has largely been unrecognized by the vector control community. The observation that insect mortality is correlated directly with crystal free energy (or inversely with crystal thermodynamic stability) was not known before our reports, and the observations for so many examples make this link between crystal energy and insecticidal activity statistically robust. Moreover, we have yet to find a compound with multiple polymorphs that is contrary to this trend. Having established a compelling link between crystal polymorphism of contact insecticides and vector control efficacy, we have commenced a comprehensive investigation of polymorphism in contact insecticides, with particular attention to their relative stabilities, both thermodynamic and kinetic. Twelve compounds have been recommended for indoor residual spraying (IRS) by the WHO [20], nine of which are crystalline at room temperature. Single-crystal structures of bifenthrin (BF), etofenprox (ET) and β-cyfluthrin (β-CF) are reported herein for the first time, as well as three new polymorphs of three other compounds: α-cypermethrin (α-CP), λ-cyhalothrin (λ-CH) and thiacloprid (TC).

Certain atoms in the molecular structure of Figure 2 are labeled (*R*) or (*S*), which is the convention for distinguishing the arrangements of chemical groups attached to a so-called stereogenic atom [21]. DM has three such stereogenic atoms. Each such atom can give rise to two stereoisomers. The number of stereoisomers is 2^N, where N is the number of stereogenic centers. Consequently, $2^3 = 8$ for DM. Organic synthesis frequently gives rise to a mixture of stereoisomers. Each would have a unique crystal structure. Racemic compounds often contain enantiomeric pairs in crystals. One stereoisomer or racemate can still be polymorphic, however. Deltamethrin, because of some fortuitous aspects of its synthesis, is generated only as the *RRS* stereoisomer (Figure 2) [7]. Because of the flexibility around the eight single bonds (C–C, and C–O), even a single, stable stereoisomeric configuration can lead to multiple polymorphs as illustrated in Figure 1C for DM, a superposition of the (*RRS*) Forms I and II.

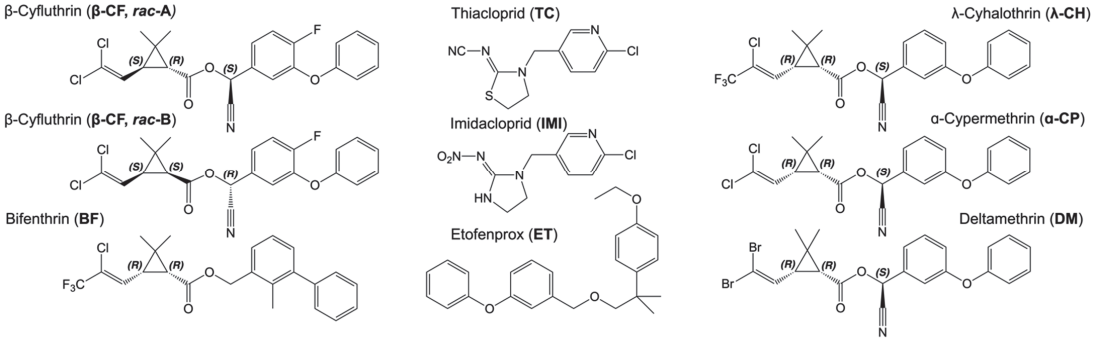

Figure 2. Molecular structures of the insecticides discussed herein. Notes on stereochemistry: Configurations of stereogenic centers are read directly in structures above from left to right throughout, a shortcut past naming conventions that are cumbersome here. β-cyfluthrin (β-CF): *SRS*-enantiomer shown in racemic mixture A (*rac*-A), and the *SSR*-enantiomer in racemic mixture B (*rac*-B). Bifenthrin (BF): *RR*-enantiomer of a racemic mixture is shown. λ-cyhalothrin (λ-CH): *RRS*-enantiomer of a racemic mixture is shown. α-cypermethrin (α-CP): *RRS*-enantiomer of racemic mixture shown. Deltamethrin (DM, *RRS* stereoisomer) is enantiomerically pure.

2. Materials and Methods

Bifenthrin (BF, CAS Number 82657-04-3) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and used as supplied. BF was grown by lowering the temperature of a supersaturated solution of ethyl acetate from 50 °C to 4 °C, the solution was kept at 4 °C until crystals were seen, at which point it was allowed to stand at room temperature.

β-Cyfluthrin (β-CF, a solid mixture comprising the racemate *RSS/SRR* (β-CF, *rac*-A) in 2:1 ratio with the diastereomeric crystal racemate *RSR/SRS* (β-CF, *rac*-B), CAS Number 1820573-27-0, was purchased from Sigma Aldrich (St. Louis, MO, USA). β-CF *rac*-A was grown by slow evaporation from a saturated methanol solution at room temperature. β-CF *rac*-A and *rac*-B were grown from mineral oil at 4 °C.

Etofenprox (ET, CAS Number 80844-07-1) was purchased from Sigma Aldrich (St. Louis, MO, USA). A single crystal of ET was retrieved directly from the bottle purchased from the manufacturer (Sigma Aldrich, St. Louis, MO, USA).

α-Cypermethrin (α-CP, CAS 67375-30-8) was purchased from Sigma Aldrich (St. Louis, MO, USA). A single crystal of α-CP was grown by cooling its melt to 75 °C on a glass slide mounted on a microscope hot stage (Mettler FP82HT) at 75 °C.

λ-Cyhalothrin (λ-CH, CAS Number 91465-08-6) was purchased from Sigma Aldrich (St. Louis, MO, USA). Crystals of λ-CH Form I were grown from the melt at room temperature. The melt of form I was seeded with α-CP to yield λ-CH Form II, which then grew from the melt at room temperature (Figure S1).

Thiacloprid (TC, CAS Number 111988-49-9) TC was purchased from Sigma Aldrich (St. Louis, MO, USA). Forms I and II of thiacloprid were grown at room temperature by slow evaporation from saturated solutions of acetone and ethyl acetate, respectively.

All solvents were purchased from Sigma Aldrich (St. Louis, MO, USA) and used as supplied. Complete descriptions of single-crystal X-ray structure analysis, powder diffraction, and spectroscopic characterization are available in the accompanying Supporting Information.

3. Results and Discussion

Bifenthrin (BF) (Figure 3A) is used against malaria and filaria vectors. It has been established that the (*RR*)-stereoisomer is 300 times more active against insects than (*SS*), which is 3–4 times more toxic to humans [22]. Crystals of a racemic mixture of BF (*RR* and *SS* stereoisomers) were grown by evaporation of an ethyl acetate solution. The crystal

structure was determined at 200 K: monoclinic space group $C2/c$, $Z = 8$, $Z' = 2$ (see Table 1). Morphologies of crystals are shown in Figure S3.

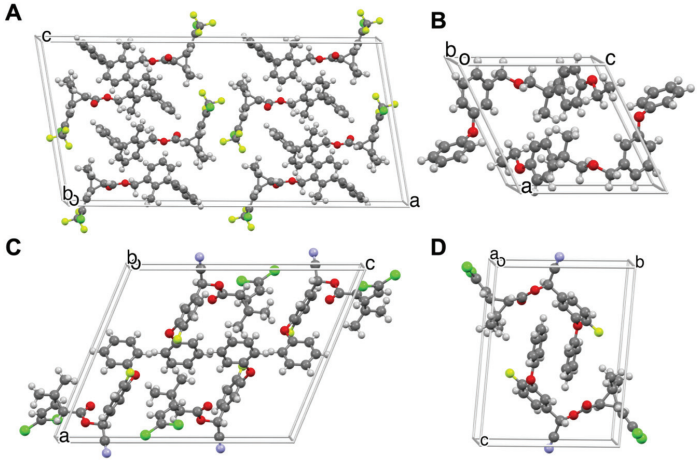

Figure 3. Crystal structures of (**A**) bifenthrin (BF), (**B**) etofenprox (ET), (**C**) *rac*-A β-cyfluthrin (β-CF), and (**D**) *rac*-B β-cyfluthrin (β-CF).

The commercially purchased form of β-cyfluthrin (β-CF), a common household insecticide, exists as a mixture comprising *rac*-A and *rac*-B (*RRS* and *SSR*) in a 2:1 ratio, respectively. Block-shaped crystals of β-CF, *rac*-A (Figure 3C), were grown from the commercial mixture by evaporation of a methanol solution in the centrosymmetric monoclinic space group $P2_1/c$, $Z = 4$, $Z' = 1$. β-CF *rac*-B (Figure 3D) was crystallized as {001} needles from a mineral oil solution stored at 4 °C in the triclinic space group $P\bar{1}$, $Z = 2$, $Z' = 1$.

Etofenprox (ET) (Figure 3B) is used to combat malaria and Zika vectors. A single crystal was selected from the manufacturer's (Sigma Aldrich, St. Louis, MO, USA) bottle and the structure was determined at 100 K. Achiral ET crystallized as {100} plates in the centrosymmetric triclinic space group $P\bar{1}$, $Z = 2$, $Z' = 1$ (Table 1).

A racemic mixture of *cis*-(*RRS*/*SSR*) α-cypermethrin (α-CP) is used in long-lasting insecticide nets and IRS formulations. Five entries appear in the Cambridge Structures Database (CNPOVN, LENDEN, LENDIR, LENDOX, SISYUO), but only CNPOVN and SISYUO contain complete structures [23,24]. Entry CNPOVN (space group $P\bar{1}$) is a racemate of the *trans*-(*RRR*/*SSS*) isomers and SISYUO (space group $P2_12_12_1$) is the *cis*-*RRS* isomer. Plates of α-cypermethrin were obtained by cooling the melt. They were refined in the centrosymmetric monoclinic space group $P2_1/n$, $Z = 4$, $Z' = 1$. Consequently, the crystals obtained from the melt correspond to a new polymorph, and the first crystal structure of the commercial form, the enantiomeric pair *cis*-(*RRS*/*SSR*), Form I (Figure 4).

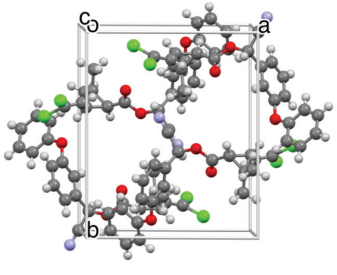

Figure 4. Crystal structure of α-cypermethrin Form I.

Cyhalothrin (CH) is a type II pyrethroid with eight possible stereoisomers. The (*SSR*) and (*RRS*) are designations for the stereoisomers of the racemic pair, which comprise a mixture known as λ-cyhalothrin (λ-CH). A structure of λ-CH was reported previously [25]. The mixture of stereoisomers, however, crystallizes as two platy forms. Form I (Figure 5A) was obtained from the melt and crystallized in the centrosymmetric monoclinic space group $C2/c$, $Z = 8$, $Z' = 1$ (See Table 1). Form II (Figure 5B), grown by seeding the melt with α-CP, also crystallizes in a centrosymmetric monoclinic space group, $P2_1/n$, $Z = 4$, $Z' = 1$, (See Table 1).

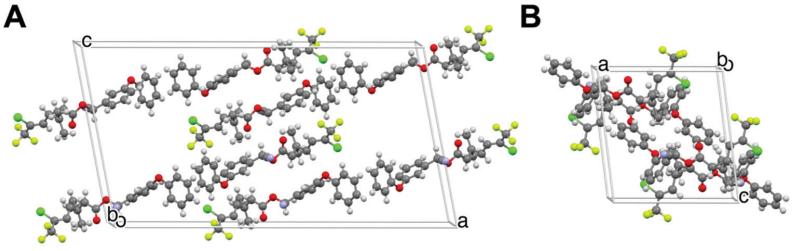

Figure 5. (**A**,**B**) Single-crystal structures of λ-cyhalothrin Forms I (**A**) and II (**B**). In (**A**), the unit cell contains 8 symmetry-related molecules. In (**B**), the unit cell contains 4 symmetry-related molecules.

Thiacloprid, TC, is a neonicotinoid insecticide like IMI but less toxic to mammals as well as honeybees [26,27]. TC crystallized from the melt as three distinct morphologies: banded spherulites, smooth spherulites, and regions with chaotic texture (Figure 6). The two spherulite morphologies were distinct by Raman microscopy (Figure S2). The chaotic texture and banded spherulites (T_m = 135 °C) corresponded to the commercially available form, designated Form I, whilst the smooth spherulites (T_m = 125 °C) corresponded to a new Form II. Form I (Figures 6 and 7A) crystallized as blocks in the monoclinic space group $P2_1/c$, $Z = 4$, $Z' = 1$ (See Table 1). The structure of Form II (Figures 6 and 7B), grown as needles, was reported previously, and confirmed here as the monoclinic space group $P2_1/c$, $Z = 8$, $Z' = 2$ [28]. The concentric bands, a consequence of helicoidal twisting of crystallites growing radially, is a common phenomenon among melt-grown molecular crystals, which we have documented thoroughly [29–33].

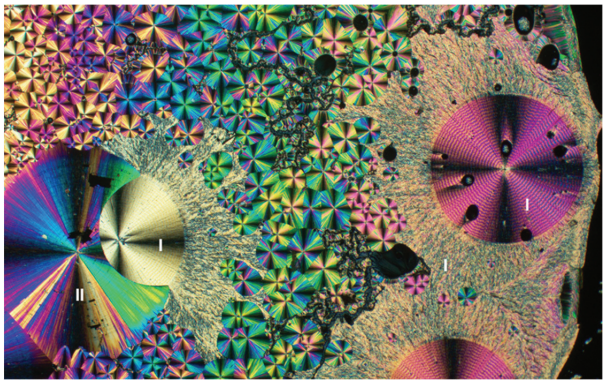

Figure 6. Thin film of thiacloprid crystals grown by cooling its melt, as viewed between crossed polarizers. Form I presents as banded spherulites and a chaotic texture, Form II as smooth spherulites.

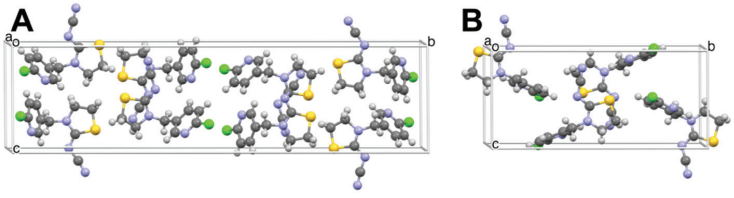

Figure 7. Single-crystal structures of thiacloprid Forms I (**A**) and II (**B**). The unit cell of Form I contains 8 symmetry-related molecules, whereas the unit cell of Form II contains 4 symmetry-related molecules.

Table 1. Single-crystal X-ray crystallography data obtained for insecticides, and corresponding experimental conditions.

Compound	Fenthrin	B-Cyfluthrin, *rac*-A	B-Cyfluthrin, *rac*-B	Etofenprox	α-Cypermethrin	λ–Cyhalothrin		Thiacloprid	
Polymorph	I	I	I	I	I	I	II	I	II
CCDC No.	2142944	2142946	2142945	2142943	2142947	2142941	2142942	2142940	2142939
Formula	$C_{23}H_{22}ClF_3O_2$	$C_{22}H_{18}Cl_2FNO_3$		$C_{25}H_{28}O_3$	$C_{22}H_{19}Cl_2NO_3$	$C_{23}H_{19}ClF_3NO_3$		$C_{10}H_9ClN_4S$	
M_w, g/mol	422.87	434.27		376.50	416.30	449.85		252.72	
Space Group	$C2/c$	$P2_1/c$	$P\bar{1}$	$P\bar{1}$	$P2_1/n$	$C2/c$	$P2_1/n$	$P2_1/c$	$P2_1/c$
Z, Z'	8, 2	4, 1	2, 1	2, 1	4, 1	8,1	4,1	4, 1	8, 2
a, Å	35.061 (3)	15.4332 (8)	6.5099 (16)	10.3004 (8)	11.497 (2)	34.273 (2)	11.8222 (9)	7.4438 (14)	7.0305 (3)
b, Å	7.1704 (5)	7.5413 (4)	11.086 (3)	10.5102 (8)	13.712 (2)	6.9368 (5)	14.3037 (11)	18.305 (3)	35.2105 (13)
c, Å	17.1168 (12)	19.3706 (10)	14.333 (3)	10.6408 (8)	12.972 (2)	18.3172 (12)	12.5427 (10)	8.2436 (15)	9.0164 (3)
α, °	90	90	94.487 (10)	86.176 (3)	90	90	90	90	90
β, °	99.999 (3)	112.348 (2)	96.984 (11)	63.403 (3)	98.349 (2)	101.2360 (10)	97.1020 (10)	95.439 (6)	98.2269 (11)
γ, °	90	90	99.455 (11)	87.263 (3)	90	90	90	90	90
V, Å^3	4237.8 (5)	2085.14 (19)	1007.7 (4)	1027.56 (14)	2023.2 (6)	4271.3 (5)	2104.7 (3)	1118.21 (4)	2209.02 (14)
D_c, g/cm^3	1.326	1.383	1.431	1.217	1.367	1.399	1.420	1.501	1.520
μ, mm^{-1}	0.222	0.343	0.355	0.078	0.344	0.230	0.233	0.504	0.510
2θ range, °	2.36–28.30	2.18–28.33	1.87–26.97	1.94–28.32	2.174–28.317	1.211–28.288	2.169–28.316	2.23–26.00	2.31–28.30
T, K	200	201	295	100	100	100	100	201	200
Total Reflections	5187	5176	4015	5085	5045	5297	5246	2714	5478
Observed Reflections	2595	3318	1642	3319	4452	4208	43842	2811	4586
No. Parameters	265	264	264	256	255	282	282	145	289
$R_1[I > 2\sigma(I)]$	0.0735	0.0630	0.1579	0.0608	0.0349	0.0463	0.0502	0.0369	0.0401
wR_2 all data	0.2601	0.1731	0.4197	0.1506	0.0945	0.1276	0.1354	0.0946	0.1052
GoF	1.033	1.054	1.043	1.022	1.067	1.022	1.007	1.087	1.032

M_w = Molecular Mass, D_c = Crystallographic Density, μ = Absorption coefficient, GoF = Goodness of fit. Thiacloprid Form II and λ-Cyhalothrin Form I were previously reported [25,28].

4. Conclusions

DM, first synthesized in 1973, was the most potent synthetic insecticide ever at the time and was heralded for its high selectivity to insects compared with mammals, a ratio of 13,000 [7]. Upon stereoselective synthesis, the solution contains the (*RRR*) and (*RRS*) stereoisomers (Figure 2). The latter crystallizes more readily, leaving the more soluble (*RRR*) isomer in solution, which can be epimerized at the cyano-bearing carbon atom to produce more (*RRS*). This is fortuitous in that (*RRS*) is the most active insecticide. Stereoisomerism is an essential feature of biological specificity.

More commonly, insecticides are supplied as mixtures of stereoisomers, which can greatly increase the complexity of crystallographic characterizations and give rise to variable crystallization outcomes (See Table S1 for crystallization conditions). The activity of crystalline contact insecticide is dictated foremost by the rate of absorption at the interface between the crystal and the target organism. Yet little attention has been paid to insecticide crystallography. This knowledge gap is exemplified by the twelve insecticides recommended for IRS by the WHO, five of which were not previously characterized crystallographically. Herein, we have reported the characterization of seven new crystallographic forms of six contact insecticides, expanding a comparatively small structural knowledge base. As mentioned above, our laboratory has demonstrated a convincing correlation between contact insecticide activity and the respective free energies of their crystal polymorphs. This behavior remains to be validated for the new characterized materials described herein.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/insects13030292/s1, Table S1: Preparation of insecticide crystals from solution crystallization, Figure S1: Heterogenous nucleation of λ-Cyhalothrin Form II on α-Cypermethrin crystals, Figure S2: Raman spectra of thiacloprid Form I and Form II, Figure S3: Single-crystal images with Miller indices.

Author Contributions: B.E., A.S. and X.Z. characterized polymorphs. B.E., A.S., X.Z. and C.T.H. performed the single-crystal analysis. B.E.K., A.S. and M.D.W. directed the project. B.E., A.S., M.D.W. and B.K wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Crystal structure data reported in this paper have been deposited at the Cambridge Crystallographic Data Centre with accession numbers found in Table 1.

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A Yeast RNA-Interference Pesticide Targeting the *Irx* Gene Functions as a Broad-Based Mosquito Larvicide and Adulticide

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Simple Summary: It is critical that we identify new methods of preventing mosquito-borne infectious diseases, which threaten millions of people worldwide. In this investigation, we describe characterization of a new insecticide that turns off the mosquito *Iroquois* (*Irx*) gene, which is required for mosquito survival. The pesticide is synthesized in yeast, which can be fed to adult mosquitoes in a sugar bait solution or to juvenile mosquitoes that eat the yeast when it is placed in water where mosquitoes breed. Although the yeast kills several different types of mosquitoes, it was not found to affect the survival of other types of arthropods that consumed the yeast. These results indicate that yeast insecticides could one day be used for environmentally friendly mosquito control and disease prevention.

Abstract: Concerns for widespread insecticide resistance and the unintended impacts of insecticides on nontarget organisms have generated a pressing need for mosquito control innovations. A yeast RNAi-based insecticide that targets a conserved site in mosquito *Irx* family genes, but which has not yet been identified in the genomes of nontarget organisms, was developed and characterized. *Saccharomyces cerevisiae* constructed to express short hairpin RNA (shRNA) matching the target site induced significant *Aedes aegypti* larval death in both lab trials and outdoor semi-field evaluations. The yeast also induced high levels of mortality in adult females, which readily consumed yeast incorporated into an attractive targeted sugar bait (ATSB) during simulated field trials. A conserved requirement for *Irx* function as a regulator of proneural gene expression was observed in the mosquito brain, suggesting a possible mode of action. The larvicidal and adulticidal properties of the yeast were also verified in *Aedes albopictus*, *Anopheles gambiae*, and *Culex quinquefasciatus* mosquitoes, but the yeast larvicide was not toxic to other nontarget arthropods. These results indicate that further development and evaluation of this technology as an ecofriendly control intervention is warranted, and that ATSBs, an emerging mosquito control paradigm, could potentially be enriched through the use of yeast-based RNAi technology.

Keywords: *Aedes albopictus*; *Aedes aegypti*; *Anopheles gambiae*; ATSB; *Culex quinquefasciatus*; insecticide; Iroquois; mosquito; RNAi; *Saccharomyces cerevisiae*; yeast

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1. Introduction

Although insect control is the principal method of mosquito-borne disease prevention, insecticide resistance [1], combined with concerns for unintended negative impacts of insecticides on nontarget species [2], threatens ongoing international mosquito control efforts. The discovery of new classes of ecofriendly insecticides and new mosquito control techniques will help to ensure the future of successful mosquito control programs and arthropod-borne disease prevention [1,3]. The development of an adequate range of new insecticide classes is dependent upon accelerating the research and development of novel active ingredients and products for mosquito control [1]. To this end, RNA interference (RNAi)-based insecticides, a new class of insecticides for mosquito control, are presently being developed and evaluated [4,5]. RNAi is a conserved innate eukaryotic regulatory pathway that functions in response to double-stranded RNA (dsRNA), serving to protect organisms from exogenous pathogenic nucleic acids through the production of small interfering RNA (siRNA). siRNA silences expression of genes that are complementary in sequence through mRNA cleavage or translation inhibition [6]. Experimental applications for RNAi have permitted the functional characterization of genes in many different organisms, including mosquitoes [5,7]. RNAi technology could potentially be translated from the laboratory to the field, where recent efforts to extend this technology for agricultural [8] and disease vector insect control are gaining traction [5,7].

In mosquitoes, laboratory screens [9,10] have resulted in the discovery of small interfering RNAs (siRNAs) which target larval lethal genes, loci that are necessary for mosquito survival during the larval stages. Several of these larvicidal siRNA target genes are also required in adult mosquitoes and can, therefore, function as both larvicides and adulticides [11,12]. A subset of the siRNAs match target sites that are conserved in *Aedes* (dengue, chikungunya, yellow fever, and Zika vector), *Anopheles* (malaria vector), and *Culex* (lymphatic filariasis and West Nile vector) mosquito species, but which have not yet been identified in other genome sequences, including humans, as well as pollinators such as honey bees [12–14]. Ongoing characterization of these interfering RNAs and larval/adult lethal loci has supported the hypothesis that interfering RNA pesticides (IRPs) will kill several different species of mosquitoes at multiple stages of the mosquito life cycle yet pose little threat to nontarget species. The present investigation further examines this hypothesis through characterization of a putative larvicidal and adulticidal IRP with a target site that is conserved in mosquito *Iroquois* (*Irx*) family genes and which lacks an identical known target site in the genomes of nontarget organisms.

Irx family genes, which encode Iroquois-class homeodomain-containing proteins, are members of the TALE subfamily and components of the *Iroquois* gene complex, which is well conserved from insects through vertebrate organisms [15]. The *Irx* complex functions to regulate transcription, controlling territory and cell fate specification decisions, pattern formation, and cell-sorting behavior [16]. *Irx* family genes were initially discovered in *D. melanogaster* (reviewed by [15]), in which the function of the *Irx* complex is required for viability [15,16], supporting the hypothesis that *Irx* silencing in mosquitoes could result in death. Here, this hypothesis was evaluated during both the larval and adult stages in several species of disease vector mosquitoes through oral RNAi experiments, which were conducted using a yeast strain that expresses short hairpin (shRNA) that silences mosquito *Irx* genes.

Laboratory evaluation of several different interfering RNA delivery mechanisms resulted in the identification of baker's yeast (*Saccharomyces cerevisiae*) as a promising method for oral transfer of interfering RNA to mosquitoes. *S. cerevisiae* is an excellent system for producing interfering RNA [4], and yeast is a potent odorant attractant for both gravid adult mosquitoes, which are lured to lay eggs in yeast-treated containers [17], as well as mosquito larvae, which readily consume larvicidal yeast upon hatching [9]. Moreover, the selection of *S. cerevisiae*, a model organism that is amenable to genetic manipulation, has facilitated generation of multiple yeast interfering RNA larvicide strains, each one targeting a different gene required for mosquito survival, resulting in the creation

of an arsenal of yeast IRPs to combat pesticide resistance [4,5]. Importantly, the insecticidal properties of the RNA are preserved when the yeast is heat-inactivated, a key finding which would potentially allow the use of dead microbials, rather than live genetically modified organisms, to control mosquitoes in the field [9]. Production of interfering RNA through yeast culturing is expected to significantly reduce the costs of this intervention at scale, and fermentation is easily expanded from small laboratory-sized shake cultures to industrial scale [4]. *S. cerevisiae*, which is not toxic to humans, is utilized worldwide for beverage and food production and has been cultivated globally for thousands of years, suggesting that this yeast technology is readily adaptable for use in resource-limited regions of the world. Yeast can also be packaged and shipped without difficulty, which can enable global distribution of yeast pesticides [4]. For these reasons, recent efforts have focused on the potential for translating use of RNAi-based yeast larvicides for mosquito control from the lab to the field [5].

In addition to characterizing a new broad-based mosquito larvicide targeting *Irx* genes, here, we explore the potential use of yeast as the insecticidal component of attractive targeted sugar baits (ATSBs) for control of multiple adult mosquito species. ATSBs, a new mosquito control paradigm, take advantage of the innate sugar feeding behavior of female and male mosquitoes that are drawn to consume a sugar source containing an insecticide. ATSBs, which are supplied through bait stations or as sprays that can be used to treat foliage or bed nets, can be used both inside and outdoors [18], and field trials indicate that this cost-effective strategy will significantly advance mosquito control efforts [19–22]. ATSBs containing a variety of different broad-based insecticides, e.g., boric acid, dinotefuran, eugenol, and garlic oil, are being used for successful targeting of *Aedes* mosquitoes [23–29]. Likewise, various *Culex* species have been effectively controlled with ATSBs that deliver insecticides such as dinotefuran, boric acid, eugenol, encapsulated garlic oil, and Spinosad [24,30–33], and ATSBs targeting *Anopheles* mosquitoes are being developed as a mechanism for addressing residual malaria transmission [20,22,34]. Although ATSBs are a highly promising technology that will greatly facilitate targeted delivery of a number of different insecticides, insecticide resistance nevertheless remains to be a concern. Notwithstanding the addition of protective barriers to bait stations [35] and attempts to limit ATSB treatments to nonflowering plants, it is hard to eradicate all risks to nontargets, such as pollinator insects, while using currently available ATSB pesticide formulations, which do not uniquely target mosquitoes [18]. Here, we investigate the potential for using yeast IRPs as a novel class of insecticides that could significantly enhance ATSB technology. In this study, we aimed to develop and characterize a yeast interfering RNA strain that targets the mosquito *Irx* gene. We evaluated the yeast as a mosquito larvicide, and then developed and tested a yeast RNAi-ATSB delivery system for targeting adult insects of the three major genera of disease vector mosquitoes. We also examined a mode of action for the yeast in the mosquito nervous system and assessed the impact of yeast treatments on nontarget arthropods.

2. Materials and Methods

2.1. Mosquito Stains and Rearing

The following strains of mosquitoes were used in this investigation: *A. albopictus* Gainesville (BEI Resources, NIAID, NIH: MRA-804, donated by Sandra A. Allan), *A. aegypti* Liverpool-IB12 (LVP-IB12), *A. gambiae* G3 (BEI Resources, NIAID, NIH: Eggs, MRA-112, furnished by Mark Benedict), and *C. quinquefasciatus* JHB (supplied by the CDC to be distributed by BEI Resources, NIAID, NIH: Eggs, NR-43025). The strains were cultured as previously described [36] in an insectary maintained under the following conditions: 26.5 °C, ~80% relative humidity, and with a 12 h dark/12 h light cycle which incorporated a 1 h crepuscular period at the beginning and end of each cycle. An artificial membrane (purchased from Hemotek Limited, Blackburn, UK) was used for delivery of sheep blood purchased from HemoStat Laboratories, Dixon, CA, USA.

2.2. Discovery of siRNA #447

siRNA #447, which contains a target sequence identified in the *Irx* genes of multiple species of mosquitoes (see details in Table S1), was initially screened in larval soaking [9,10,37] and adult microinjection assays [11,12] that were completed in *A. aegypti* as previously discussed. Soaking experiments, which were performed in two replicate trials, were completed using 20 first instar (L1) larvae which were soaked in 20 uL of 0.5 µg/µL of siRNA #447 or control siRNA (custom synthesized by Integrated DNA Technologies, Coralville, Iowa) for 4 h. Following soaking treatments, the larvae were reared and evaluated as detailed in the World Health Organization (WHO) larvicide testing guidelines [38], and data were evaluated with the Fisher's exact test. siRNA sequences were as follows: siRNA #447: 5′–AAAAAACCAAACGGGCAGCGACUGU–3′, control: 5′–GAAGAGCACUGAUAGAUGUUAGCGU–3′ [39]. For assessing the adulticidal activity as previously described [11,12], 20 3 day old non-blood fed adult females per treatment were anesthetized using carbon dioxide and microinjected in the thoracic region with 250 nL of 9 µg/µL *Irx*.447 or control siRNA (Integrated DNA Technologies, Coralville, IA, USA), after which time the mosquitoes were put in a cage for recovery. Adult mortality was subsequently evaluated every day for the next 6 days.

2.3. siRNA-ATSB Trials in Adults

ATSB trials with siRNA were completed as previously described [11,12] using 64 µL of 2.5 µg/µL siRNA in 10% sucrose solution containing 4.5% of red tracer dye (McCormick) which was dispensed from a cotton wick placed in a cut 0.2 mL microcentrifuge tube hung in a 3.75 L mosquito cage (Berry Global, Evansville, IN) located in the insectary. Three replicate trials were performed using 25 4–5 day old non-blood-fed adult females which were sugar-starved for 48 h prior to sugar bait feedings that were initiated at dawn and conducted for 4 h. Feeding was verified on the basis of the presence of red dye in the abdomen. Females that had sugar fed were evaluated daily for 6 days, after which time feeding rates were statistically evaluated using the G-test of independence, and the log-rank test was used for comparison of survival rates among treatments.

2.4. Yeast Larvicide Strain Generation and Culturing

Custom DNA oligonucleotides encoding an shRNA expression cassette that corresponds to *Irx*.447 target site 5′–AAACCAAACGGGCAGCGACTG–3′ were purchased from Invitrogen Life Technologies (Carlsbad, CA) and used in the generation of transformants bearing shRNA expression cassettes stably integrated at both the *ura3* and *trp1* sites of the *S. cerevisiae CEN.PK* strain [40] as previously described [9]. This yeast strain, which is referred to as *Irx*.447 yeast, as well as a similar control shRNA expression strain constructed in a previous study [9], was cultured for preparation of 50 mg tablets of dried inactivated yeast larvicide as described [41]. For ATSB trials, yeast was cultured in a similar manner, except that it was lyophilized in a Labconco FreeZone 6 L Console Freeze Dryer after culturing and pelleting, and then used for ATSB production and trialing as detailed below.

2.5. Larvicide Trials

Evaluation of larvicides was performed according to the WHO testing guidelines [3] as described [41]. Each of 18 replicate container trials was performed using 20 first-instar larvae (n = 360 larvae total per treatment) that were reared in 50 mL volumes of distilled water placed in 500 mL sized containers, along with a single 50 mg yeast tablet (either *Irx*.447 or control) that was available at the onset of each trial. Larvae were appraised throughout the trial period. At the end of the trial, larval mortality percentages were transformed using arcsine transformation as recommended prior to analyzing data with the Student's t-test. Dose–response curves were generated and analyzed as previously described [9] using varying amounts of insecticidal and control interfering RNA yeast to prepare tablets with different doses of larvicide that were evaluated in larvae.

Semi-field evaluations of larvicides were performed according to the WHO larvicide testing guidelines [38] on an outdoor rooftop laboratory during July and August 2019 in Notre Dame, IN as previously described [13,14]. During the trial period, the average relative humidity was 75% ± 15%, and outdoor temperatures ranged from 9–35 °C, with a mean daytime temperature of 24 ± 5 °C and a mean nighttime temperature of 19 ± 4 °C. Each of 19 replicate container trials was performed using 20 LVP-IB12 strain *A. aegypti* mosquitoes that were placed in 7.5 L buckets (diameter = 23 cm, height = 25 cm) with 3.5 L of water and a 50 mg larvicidal or control yeast tablet. After the trials, the larval mortality rates in larvicide- or control-treated containers were transformed with arcsine transformation, and data were evaluated using a Student's *t*-test.

2.6. Yeast ATSB Assays

For performance of *A. aegypti* simulated field trials, which were conducted in the insectary, yeast ATSB was prepared by homogenizing 40 mg of lyophilized yeast (*Irx*.447 or control) which contained 0.1% benzoic acid preservative with a 5% sucrose solution containing 0.05% Phytagel brand gellan gum (Sigma Aldrich, St. Louis, MO, USA; used to hinder ATSB desiccation) that was marked through the addition of 4.5 µL of red dye (McCormick's) to a total volume of 100 µL that was placed in a 1.5 mL microfuge tube. For experiments conducted with *A. gambiae*, which are smaller than *Aedes* mosquitoes, the amount of yeast was halved to 20 mg per 100 µL of ATSB. Feeders were prepared by scoring the bottom of the 1.5 mL microfuge tube, which was then capped and perforated prior to hanging the yeast wick feeder at the top of the experimental cage, which was placed in the insectary. A total of 25 non-blood-fed 5–6 day old adult females that were sugar-starved for 2 days were placed in 3.75 L insect cages (Berry Global, Evansville, IN, USA), where they were permitted to eat from the two feeders for 4 h. Negative controls included ATSB with control yeast or ATSB with no yeast. After completing three replicate trials, mosquito feeding rates were assessed using the G-test of independence, and survival rates were evaluated using ANOVA. *Irx*.447 dose–response curves were generated and evaluated as described for the yeast larvicides in Section 2.5.

2.7. Whole-Mount In Situ Hybridization

The Patel [42] protocol was used to prepare riboprobes used to verify silencing of *Irx* genes (Table S1). Probes corresponding to the *A. aegypti POU domain protein 2* (*pdm2, AAEL017445*) and *A. gambiae pdm2* (*AGAP009500*) genes were also synthesized and used to assess the *Irx*.447 mode of action. The probes were used for detection of *Irx* and *pdm2* transcripts in adult female brains through in situ hybridization experiments, which were conducted as previously described [43]. Three biological replicate experiments were performed, and results were viewed and imaged with a Zeiss Axioimager (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) equipped with a Spot Flex imager (Diagnostic Instruments, Inc. Sterling Heights, MI, USA). Images were processed using FIJI ImageJ software [44], which was used to assess mean gray value (average signal intensity over a specified area) data, allowing for quantification of digoxigenin-labeled transcripts and data analysis using the Student's *t*-test as described [45].

2.8. Evaluation of Nontarget Species

Yeast IRP toxicity was evaluated as described [11,12] in *Daphnia magna, Drosophila melanogaster,* and *Tribolium castaneum.* Toxicity assays were performed in *Hippodamia convergens* and *Oncopeltus fasciatus* according to the procedures described below.

O. fasciatus adults were acquired from Carolina Biologicals (Burlington, NC, USA) and cultured as described by the provider. For toxicity tests, which were performed in duplicate, a slurry of 200 µL of 10% sucrose combined with red marker dye and 50 mg of either *Irx*.447 or control interfering RNA yeast was provided to 20 adults (the total amount of yeast consumed per insect was the same as that used in mosquito assays). A 0.5 mL tube with a wick was suspended from the cage (which was maintained at room temperature,

21 °C) for delivery of the slurry to the insects throughout a six day trial period. Feeding was verified through observation of feeding bouts, as well as through observation of red marker dye in the insect feces. Survival data were analyzed using Fisher's exact text.

H. convergens adults (Carolina Biologicals, Burlington, NC, USA) were reared in cages maintained at room temperature (21 °C) as directed by the supplier. Toxicity assays were conducted as described above for *O. fasciatus*, but were completed using 10 insects that fed on yeast ATSB which had been provided in a small dish throughout the trial period.

3. Results and Discussion

3.1. Silencing Irx Kills A. aegypti Mosquitoes

Irx.447 siRNA matches a conserved target sequence in *Irx* family genes of multiple mosquito species (Table S1) [46]. An identical sequence was not identified in the sequenced genomes [47] of other organisms (Table S1). The potential for *Irx*.447 siRNA to function as an insecticide was first evaluated in *A. aegypti*, in which siRNA soaking treatments resulted in significant larval mortality (Table 1). Significant mortality was also seen in *A. aegypti* adult females that were microinjected with *Irx*.447 siRNA in the adult thorax (Table 1). Given the results of these microinjection experiments, the potential for delivery of *Irx*.447 IRPs through ATSBs was then evaluated in a simulated field study conducted in the insectary in which a previously described sugar bait delivery system [11,12] was used for oral transfer of the *Irx*.447 siRNAs to adult females. Feeding rates of *A. aegypti* females following 4 h of exposure to *Irx*.447 sugar bait or sugar bait mixed with control siRNA (which has no known target in mosquitoes [39]) are shown in Table S2 and were similar to those observed in comparable trials with other siRNA adulticides [11,12]. *A. aegypti* feeding rates were not significantly different among the treatments ($p > 0.05$). Although no significant mortality was observed in adult female mosquitoes that consumed sugar bait alone or containing control siRNAs, significant mortality, 75% ± 3%, was noted in adult female mosquitoes that consumed *Irx*.447 siRNA ATSB (Table 1). These results in *A. aegypti* indicated that *Irx*.447 siRNA is an insecticide that has both larvicidal and adulticidal properties.

Table 1. *Irx*.447 siRNA treatments result in *Aedes aegypti* larval and adult mortality.

Trial	% Mortality	*n*	*p*-Value
Larval soaking			
Control siRNA	5 ± 5 *	40	<0.001
lrx siRNA	72.5 ± 2.5	40	
Adult microinjection			
Control siRNA	5	20	0.0092
lrx siRNA	40	20	
ATSB feeding			
Control siRNA	8 ± 5	37	<0.001
lrx siRNA	75 ± 3	42	

* Mortality rates and standard errors of the mean (SEM), *n* numbers, and the *p*-value found in Fischer's exact tests comparing *Irx*.447 siRNA-treated vs. control-treated *A. aegypti* are shown.

3.2. Delivery of the Yeast Pesticide as an ATSB

The larvicidal and adulticidal activity of *Irx*.447 siRNA (Table 1) suggests that this insecticide could potentially be used to control mosquitoes. However, the present high costs of siRNA synthesis could impede broad deployment *Irx*.447 siRNA insecticides [5]. As noted above, this has been addressed through use of an *S. cerevisiae* shRNA expression system, which has been developed for potential use in larval control programs [4], but could potentially be deployed for use as an ATSB. In this investigation, development and evaluation of RNAi yeast targeting *Irx* facilitated examination of the hypothesis that yeast IRPs can be utilized as both larvicides and adulticides. *S. cerevisiae*, in which shRNA corresponding to the *Irx*.447 siRNA (hereafter referred to as *Irx*.447 yeast) was expressed

through the stable integration of two *Irx*.447 shRNA expression cassettes, was used. PCR amplification of cDNA corresponding to the 3′ end of the hairpin and the terminator sequence resulted in a band of the expected ~100 bp size (Figure 1A).

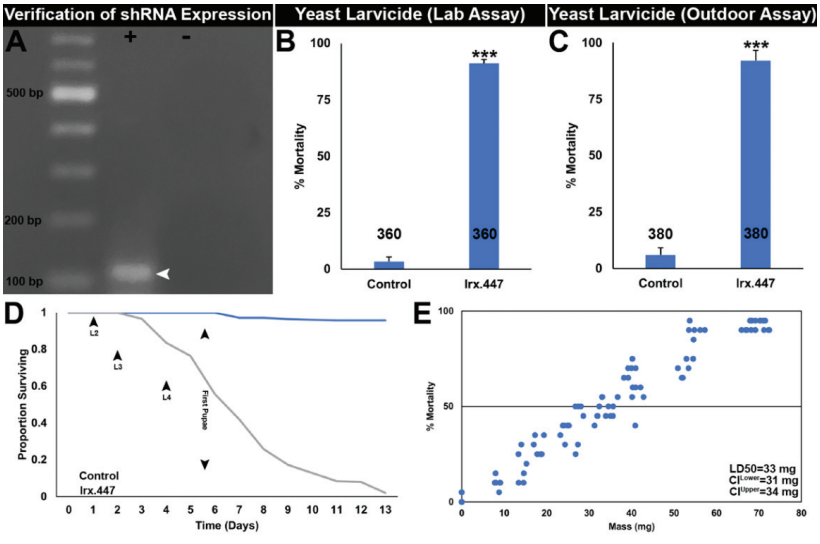

Figure 1. *Irx*.447 yeast consumption results in *A. aegypti* larval death. (**A**) A ~100 bp band amplified with primers corresponding to *Irx*.447 is seen in the lane marked by + in an agarose gel stained with ethidium bromide; cDNA prepared from *Irx*.447 yeast total RNA was the template in these reactions. No amplicon was detected in a negative control PCR reaction which lacked a cDNA template (marked by a minus symbol). A representative gel from two comparable biological replicate assays is displayed; irrelevant lanes were cropped from the image. (**B**) Consuming Rbfox1.457 yeast throughout larval development caused significant larval death in laboratory insectary trials, as well as in (**C**) semi-field outdoor trials conducted on *A. aegypti* larvae placed in 7.5 L buckets containing 3.5 L water. In (**B**,**C**), data combined from multiple replicate trials (each with 20 larvae) are displayed as mean percentages of larval mortality. (**D**) An *A. aegypti* larval survival curve corresponding to the data in panel (**B**) is shown. (**E**) A dose–response curve illustrates a positive correlation between *A. aegypti* larval mortality and the amount of *Irx*.447 yeast contained in larvicide tablets in which control and larvicidal yeast were mixed in varying proportions; each data point in (**E**) corresponds to the percentage mortality found in a single-container assay with 20 larvae; LD_{50} values are indicated. In (**B**,**C**), *n* numbers are shown under each bar in the graphs, and error bars denote SEMs; *** $p < 0.001$ (Student's *t*-test).

Prior to evaluation of the putative adulticidal activity of IRP.447 yeast, the insecticidal activity of the *Irx*.447 yeast strain was verified in larvae, in which it induced 91% ± 2% larval mortality in indoor trials (Figure 1B; $p < 0.001$ vs. control interfering RNA larvicide treatment) and 92% ± 5% larval mortality in outdoor semi-field trials (Figure 1C; $p < 0.001$ vs. control interfering RNA larvicide treatment). Although control-treated larvae survived through adulthood, most *Irx*.447 larvae died during the fourth larval instar (a survival curve is shown in in Figure 1D). Higher dosage of *Irx*.447 increased the rates of larval mortality (Figure 1E), with the LD_{50} determined to be 33 mg. These results, combined with previous studies [9,11–14], demonstrate that yeast IRPs function as potent larvicides and add *Irx*.447 to the growing arsenal of yeast IRPs.

Based on the successful verification of *Irx*.447 insecticidal activity in larvicide trials (Figure 1), the yeast was used to develop an *Irx*.447 yeast ATSB, which was evaluated in adult mosquitoes in simulated field trials conducted in the insectary. Yeast ATSB feeding

rates were nearly doubled with respect to siRNA-ATSBs (Table S2), with *Irx*.447 yeast consumption verified in 100% of adult female *A. aegypti* mosquitoes (Table S2). This resulted in 77% ± 2% mortality (Figure 2A, $p < 0.001$ compared to control yeast in 5% sugar bait), which is comparable to the mortality rates observed in *Irx*.447 siRNA ATSB trials (Table 1). Although the mortality rates induced by *Irx*.447 yeast ATSBs are slightly less than what has been observed for siRNA-ATSBs targeting *dop1* and *Shaker* [11,12], the increased feeding rates associated with the yeast ATSBs, as well as the anticipated decreased production costs of yeast, make it an appealing delivery system. *Irx*.447 yeast ATSB treatments killed a majority of *A. aegypti* adult female mosquitoes within 6 days following ATSB consumption (Figure 2B). The percentage of *A. aegypti* female mortality correlated with the concentration of *Irx*.447 yeast in the ATSB (Figure 2C), with the LD_{50} value determined to be 0.18 μg/μL ATSB.

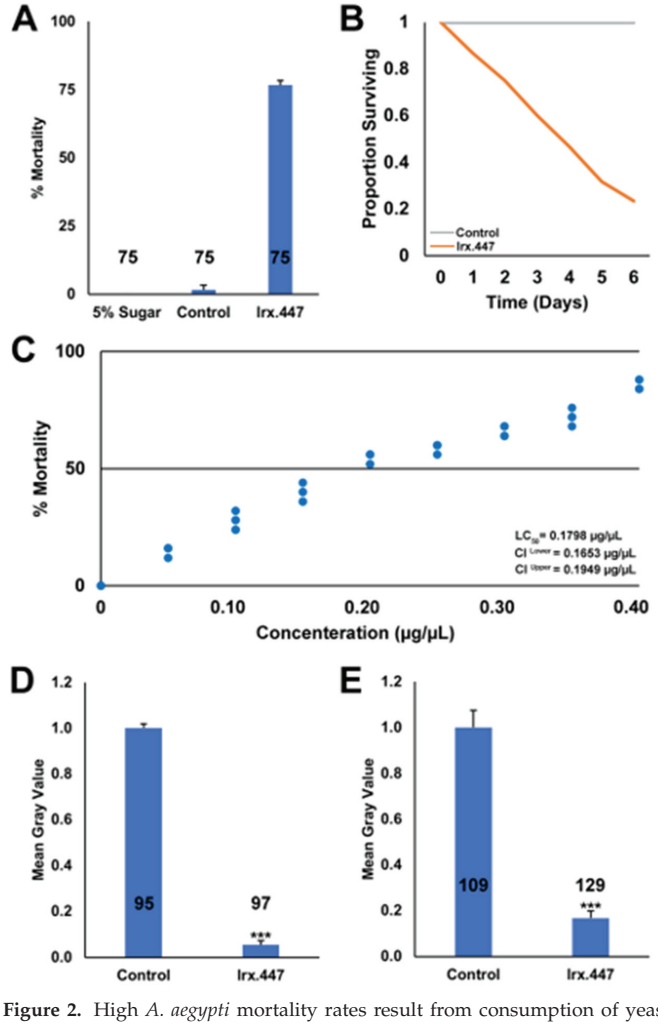

Figure 2. High *A. aegypti* mortality rates result from consumption of yeast RNAi-based ATSBs targeting *Irx*. (**A**). Significant mortality is observed following consumption of heat-inactivated *Irx*.447 yeast delivered to *A. aegypti* adult females as an ATSB. (**B**). Survival curves for adult females that consumed *Irx*.447 or control yeast sugar bait (in panel **A**) are displayed. (**C**). A dose—response curve

illustrates the concentration of *Irx*.447 yeast provided in the ATSB vs. the percentage mortality of *A. aegypti* adult females; each point corresponds to an ATSB trial conducted on 25 adult females. LD_{50} values are indicated. *Irx*.447 ATSB consumption by *A. aegypti* adult females resulted in significantly reduced levels of *Irx* (**D**) and *pdm2* (**E**) transcripts in the brain, as evidenced by mean gray value analyses. Throughout this figure: *n* numbers are displayed under each bar in the graphs, and error bars denote SEMs; *** $p < 0.001$ (Student's *t*-test).

The mode of action for *Irx*.447 IRPs was next examined. In *D. melanogaster,* the *Irx* family genes encode transcriptional regulators that activate expression of proneural genes of the *achaete–scute* complex (AS–C) [48]. Given that the role of *Irx* transcriptional control is conserved in vertebrates [15], in which the *Irx* complex regulates proneural gene expression in the nervous system [15,49], it seemed likely that this transcriptional regulatory function would also be conserved in mosquitoes. In support of this, silencing of *Irx* transcripts (Supplementary Figure S1(A1,A2), Figure 2C, $p < 0.001$ vs. control-treated females) resulted in a significant decrease in transcript levels of the proneural gene *pdm2* in the adult brain of *A. aegypti* females (Supplementary Figure S1(B1,B2), Figure 2D, $p < 0.001$ vs. control-treated females). Combined, these results suggest that loss of *Irx* function impacts expression of critical proneural gene function in the nervous system, resulting in mosquito death.

These data indicate that *Irx*.447, an insecticide with a mode of action that differs from that of existing pesticides, could help combat insecticide resistance. These results, combined with other recent IRP ATSB studies [11,12], suggest that it may be useful to develop yeast strains that express multiple shRNAs, each targeting different genes. For example, yeast that expresses *Irx*.447 shRNA in conjunction with other newly characterized broad-based larvicidal and adulticidal shRNAs, such as dop1.462 [11] and/or Sh.463 [12] shRNAs, could be constructed. Combining two or more different shRNAs, each with a different mode of action, could be useful for managing resistance to any single shRNA [50]. Interestingly, although mixtures of some pesticides can significantly increase costs [1], expressing two or more different shRNAs in a single strain would not be expected to significantly impact the cost of yeast IRP production or application, as both insecticides would be simultaneously produced during yeast cultivation and applied together, another advantage of yeast IRP technology.

3.3. Irx.447 Yeast Selectively Kills Aedes, Culex, and Anopheles Mosquitoes

An identically conserved *Irx*.447 target site is found in the sequenced genomes of many species of mosquitoes, including multiple *Anopheles* mosquitoes, *A. albopictus*, and *C. quinquefasciatus* (Table S1). It was hypothesized, on the basis of the outcomes of the ATSB and larvicide trials in *A. aegypti* (Figures 1 and 2), that *Irx*.447 yeast may act as a broad-range mosquito IRP that can kill at multiple life stages in different species of mosquitoes. In support of this hypothesis, *Irx*.447 yeast ATSB was assessed in *A. gambiae* female adults, in which 100% feeding rates (Table S2) resulting in 93% $\pm$ 1% adult mortality were observed (Figure 3B, $p < 0.001$ vs. control-yeast treated adults, in which no significant death was detected). *Irx* silencing in *A. gambiae* (Supplementary Figure S2(A1–A3)) resulted in significantly reduced *pdm2* transcript levels in the adult brain (Supplementary Figure S2(B1–B3)). Consumption of *Irx*.447 yeast ATSB also induced significant mortality in *A. albopictus* (Figure 3A, $p < 0.001$ vs. control-yeast treated adults), in which 86% $\pm$ 5% adult female mortality was observed, as well as an 84% $\pm$ 3% mortality rate in *C. quinquefasciatus* (Figure 3C, $p < 0.001$ vs. control-yeast treated adults). As with *A. aegypti* and *A. gambiae,* 100% adult female feeding rates were observed in *C. quinquefasciatus,* while feeding rates in *A. albopictus* were 87 $\pm$ 1% (Table S2).

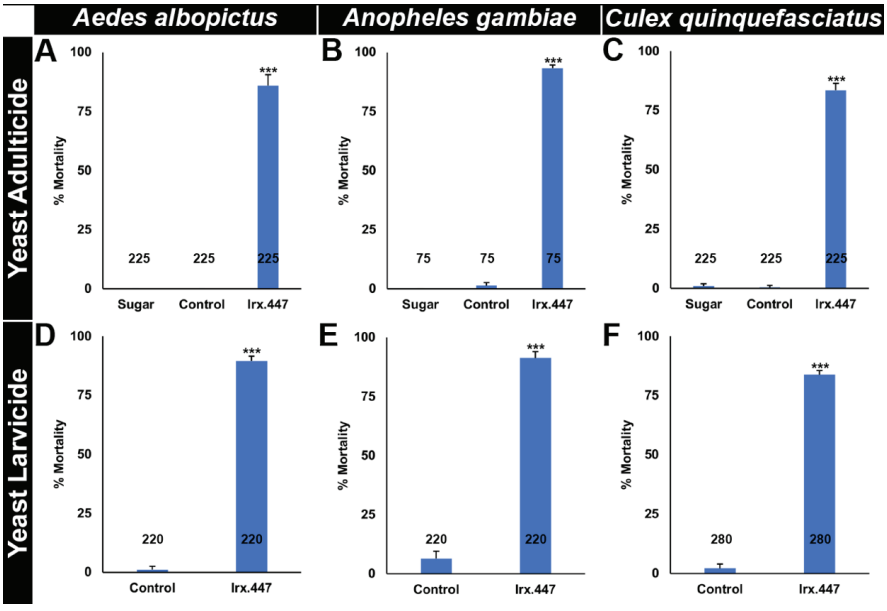

Figure 3. *Irx*.447 yeast is a broad-based insecticide. Oral consumption of dried inactivated *Irx*.447 yeast by larvae (**A**–**C**) or by adult females as an ATSB (**D**–**F**) induces significant mortality rates in *A. albopictus* (**A**,**C**), *A. gambiae* (**B**,**D**), and *C. quinquefasciatus* (**C**,**F**). Throughout the figure, data are shown as mean mortalities; error bars denote SEMs; *n* numbers are found below each bar in the graphs. Data were evaluated with the Student's t-test (**A**–**C**) or with ANOVA (**D**–**F**); *** $p < 0.001$ vs. control.

These high feeding rates (Table S2) suggest that yeast RNAi-based ATSBs may function well in the field, where many mosquito odorant attractant cues are present. The high levels of adult lethality observed in *Aedes*, *Anopheles*, and *Culex* mosquitoes also illustrate the promising nature of these insecticides. It will be both interesting and critical to evaluate yeast IRP-ATSB efficacy in the field, as it relates to the relative attractiveness of the baits, as well as to assess the residual activity of these pesticides upon exposure to outdoor elements. Future field trials to assess these questions are, therefore, planned, and methods for further preserving the yeast IRP activity, perhaps through formulations that enhance the stability of IRPs in a variety of different environmental conditions, both before and during use, may prove to be critical given that the ATSBs will need to be shipped, stored, and utilized in the tropics, where the formulations will need to persist through exposure to high heat. Yeast encapsulation could also enable the development of controlled and extended insecticide release formulations. Such formulations will likely be critical for the development of commercial products, which are often expected to have residual activities of several months [4].

Irx.447 yeast treatments also resulted in 90% ± 2% *A. albopictus* larval mortality (Figure 3D, $p < 0.001$ vs. control yeast treatment), 91% ± 3% *A. gambiae* larval mortality (Figure 3E, $p < 0.001$ vs. control yeast treatment), and 84% ± 2% *C. quinquefasciatus* larval mortality (Figure 3F; $p < 0.001$ vs. control yeast treatment). Given that larviciding is an essential component of mosquito control programs for some species of *Aedes* and *Culex* mosquitoes, the prospect of including a new class of RNAi-based larvicides to these programs is of utility and might help to address ongoing issues with resistance to existing classes of mosquito larvicides [3,51]. Although *Anopheles* mosquito control programs typically focus on adult mosquitoes, efforts to address residual transmission will need to incorporate additional mosquito control technologies [52]. The WHO [53] recommends larviciding for control when *Anopheles* breeding sites are fixed, few, and findable. Larvicid-

ing can be advantageous under certain conditions, depending on the target and the local circumstances [1]. For example, recent studies have reported that long-lasting Bti larvicides are useful for control of *A. funestus* and *A. gambiae* larvae [54–57]. *Anopheles stephensi*, a more urbanized malaria vector mosquito, can share breeding containers with *A. aegypti* [58]. It may, therefore, be possible to use the *Irx*.447 larvicide, which has a conserved target site in both mosquitoes (Table S1), to kill larvae of both species in these containers.

The ability of these insecticides to kill both larvae and adults opens opportunities to design integrated RNAi mosquito control programs in which a combination of methods, such as larvicidal treatment of breeding sites with interfering RNA larvicides, larval lethal lure-and-kill interfering RNA ovitraps [17], and RNAi-ATSBs is used simultaneously. Recent studies have uncovered a high level of acceptance of yeast RNAi-based larvicides and ovitraps among stakeholders in Trinidad and Tobago [59,60]. An engagement study in Tanzania [61] evaluated stakeholder acceptance of ivermectin-based ATSBs. The study concluded that further sensitization at the community level will be critical for educating stakeholders regarding the mode of action and use of this intervention, as most community stakeholders were not yet familiar with the ATSB paradigm. It will be interesting to gauge the acceptance of RNAi yeast-based ATSBs among stakeholders in Trinidad and elsewhere, and such studies are planned. The findings of the investigation in Tanzania [61] suggest that such studies, as well as educational campaigns that introduce stakeholders to yeast IRP ATSB technology, are essential.

Although pyrethroids, which have relatively low toxicity in humans, have been the chemicals of choice for public health control districts for several decades, widespread pyrethroid resistance threatens mosquito control strategies, necessitating the identification of novel classes of pesticides with high safety profiles [1]. In addition to evaluating the efficacy of *Irx*.447, the safety profile of this pesticide was assessed by performing toxicity screening assays in several nontarget organisms. Such assays are critical, as in silico tests are helpful but cannot exclude the possibility of off-target impacts, as one cannot predict a priori whether sites with similar, albeit not identical sequence similarity, could potentially be targeted [62]. Although *Irx*.447 yeast IRP has both mosquito larvicidal and adulticidal activities in multiple species of mosquitoes (Figures 1–3), the yeast IRP did not impact survival of a group of select nontarget arthropods that were evaluated in this investigation (Table 2), including the water flea *D. magna*, the fruit fly *D. melanogaster*, the lady beetle *Hippodamia convergens*, the milkweed bug *Oncopeltus fasciatus*, and the flour beetle *T. castaneum*. These data suggest that *Irx*.447 yeast present insignificant or no threats to nontarget species, but it will of course be important to further corroborate these initial safety profile data through pursuit of expanded toxicity testing. These tests should be performed with commercial-ready yeast formulations and involve evaluations in additional species, including pollinators and vertebrate organisms, to develop a portfolio for submission to regulatory agencies.

Table 2. Viability of nontarget arthropods following consumption of *Irx*.447 yeast.

Test organism	*n*/ Treatment	% Survival	
		Control Yeast	*Irx*.447 Yeast
D. melanogaster larvae	60 *	100 ± 0	100 ± 0
D. melanogaster adults	60	100 ± 0	100 ± 0
Tribolium adults	40	100 ± 0	100 ± 0
Oncopeltus fasciatus adults	20	80 ± 7	90 ± 14
Hippodamia convergens adults	20	100 ± 0	100 ± 0
Daphnia magna adults	40	100 ± 0	100 ± 0

* Survival was assessed after consumption of *Irx*.447 yeast or control interfering RNA yeast delivered as an ATSB to the indicated arthropods. Mean percentages of survival with SEMs, as well as *n* numbers corresponding to the number of animals treated, are indicated. Fisher's exact test comparisons did not reveal any significant differences in survival between insecticide-treated and control interfering RNA-treated arthropods.

4. Conclusions

Although mosquito control is a central and crucial component of mosquito-borne disease prevention strategies, insecticide resistance threatens current and future gains in the war against disease vector mosquitoes, and the identification and characterization of new active ingredients and products for mosquito control is critical [1]. The results of this investigation provide further support for the hypothesis that *Irx*.447 kills multiple species of mosquitoes at different life stages (Figures 1–3) yet poses little threat to nontarget species (Table 2). These data, combined with other recent studies [5], suggest that RNAi-based yeast pesticides should be further developed as a novel class of insecticides for mosquito control. Characterization of *Irx*.447 yeast demonstrated that it functions as a dual adulticidal and larvicidal IRP with activity in *Aedes, Anopheles,* and *Culex* mosquitoes (Figures 1–3), which possess a conserved *Irx* target site for this insecticide. A loss of *pdm2* transcript expression in the mosquito nervous system, which correlated with silencing of *Irx* (Figure 2D,E, Supplementary Figures S1 and S2), suggests that mortality associated with this insecticide results from disruption of proneural gene expression. Use of *Irx*.447 could facilitate the management of insecticide resistance through the addition of an insecticide with a mode of action that differs from that of existing pesticides [1].

The elimination of mosquito-borne diseases will likely require the implementation of new vector control interventions that will complement existing control measures. Thus, in addition to new insecticide classes, new paradigms will be important additions to integrated resistance management strategies [1]. To this end, the present investigation provided evidence that *Irx*.447 yeast can be successfully delivered to adult mosquitoes as an ATSB (Figures 2 and 3), a sugar-baited trap, and a new paradigm for vector control [1]. These findings suggest that further development of yeast interfering RNA pesticides, the production of which is likely to be both affordable and scalable [4], should be pursued for use in ATSBs. Confirmation of *Irx*.447 yeast ATSB activity in simulated field trials performed using bait stations in cages (Figures 2 and 3), as well as the analysis of *Irx*.447 yeast activity in outdoor semi-field larvicide trials (Figure 1C), indicates that these new RNAi-based technologies could potentially be useful in the field, a prospect that will be evaluated in future large-scale field trials which will be accompanied by stakeholder engagement activities and educational campaigns. Such trials will require scaled yeast production in larger fermentation-sized cultures, suggesting that the production of commercial strains that withstand fermentation, as well as the piloting and optimization of scaled yeast IRP production, would be advantageous [4].

Supplementary Materials: The following are available online at https://www.mdpi.com/2075-4450/12/11/986/s1: Table S1. The *Irx*.447 target site conserved in mosquitoes was not found in non-mosquito genomes [47]; Table S2. Mosquito ATSB feeding rates observed in laboratory simulated field trials; Figure S1. *Irx*.447 yeast ATSB induces target gene silencing and reduces *pdm2* expression in *A. aegypti;* Figure S2. *Irx*.447 yeast ATSB induces silencing of the *Irx* target gene and significantly reduces *pdm2* expression in *A. gambiae.*

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Conflicts of Interest: M.D.-S., N.W., and D.W.S. are inventors on US Patent Application No.: 62/361,704, European Application No. 17828458.4, but this application did not affect their data interpretation and does not influence their adherence to journal policies on sharing data and materials. All other authors declare no conflict of interest.

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Review

Plant-Based Bioinsecticides for Mosquito Control: Impact on Insecticide Resistance and Disease Transmission

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Simple Summary: Mosquito-borne diseases cause millions of deaths each year. There has been an increase in the use of insecticides to combat disease transmission caused by mosquitoes. Synthetic insecticides have been effectively used to protect humans from mosquito bites through insecticide-treated mosquito nets, fabrics, and indoor sprays. Despite the considerable progress made in reducing mosquito borne diseases, extensive usage of insecticides has caused serious health problems to humans and animals, insecticide resistance or insensitivity in mosquitoes, and environmental damage. A success in the fight with mosquito disease transmission can only be accomplished by adequate and effective implementation of insecticide resistance monitoring and management programs globally. For this purpose, extensive research focuses on exploring insecticide resistance mechanisms in mosquitoes and how they get resistant to chemical applications over time. The search also focuses on novel compounds that are more effective, safer, and eco-friendly for improved management of mosquito vectors. In this review, we provide the current literature on the synthetic insecticides and how mosquitoes develop resistance to them, with further emphasis on bioinsecticides that could replace conventional synthetic insecticides. In this context, plant-based compounds are explained in detail with their potential applications to control mosquitoes.

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Abstract: The use of synthetic insecticides has been a solution to reduce mosquito-borne disease transmission for decades. Currently, no single intervention is sufficient to reduce the global disease burden caused by mosquitoes. Problems associated with extensive usage of synthetic compounds have increased substantially which makes mosquito-borne disease elimination and prevention more difficult over the years. Thus, it is crucial that much safer and effective mosquito control strategies are developed. Natural compounds from plants have been efficiently used to fight insect pests for a long time. Plant-based bioinsecticides are now considered a much safer and less toxic alternative to synthetic compounds. Here, we discuss candidate plant-based compounds that show larvicidal, adulticidal, and repellent properties. Our discussion also includes their mode of action and potential impact in mosquito disease transmission and circumvention of resistance. This review improves our knowledge on plant-based bioinsecticides and the potential for the development of state-of-the-art mosquito control strategies.

Keywords: bioinsecticide; disease transmission; insecticide-resistance; mosquito-borne disease; mosquito control; natural compounds; phytochemical

1. Introduction

Mosquitoes have been a big burden to human health for a long time. These insects can invade in different geographic locations and new habitats through global trade and travel [1] which causes millions of people be at risk of the diseases they transmit. In 2019, an estimated 229 million cases and 409 thousand deaths for malaria and 56 million cases for dengue have been reported worldwide [2,3]. While malaria case incidences were reported to decline, the number of malaria endemic countries has increased in the

period 2000–2019 [2]. The global incidence of dengue is thought to be increased about thirty times over the last fifty years with emergencies in new countries [4–6]. A recent study also indicates that mosquito species will continue to spread globally over the coming decades, which may cause about 50% of the world's population at the risk of mosquito-borne viral disease transmission by 2050 [7]. Even a more serious problem is at our doorstep as the climate change is expected to increase the burden of mosquito-borne diseases despite the ongoing disease control interventions [8,9].

The most common way of keeping mosquitoes away from their human hosts is to use synthetic insecticides in mosquito nets, fabrics, and indoor sprays. The usage of chemical strategies has brought hope in controlling disease transmission in endemic regions, but emergence of insecticide resistance has been a major problem in reducing the disease burden. The uncontrolled usage of insecticides has led to reemergence and increase in mosquito populations over the years. Between the years 2010–2019, about 28 malaria endemic countries (out of 82) have detected resistance to all four classes of the most commonly used insecticides, and 73 have detected resistance to at least one insecticide class, an issue that continues to increase globally [2]. Thus, insecticide resistance is now considered a serious threat to control mosquito invasion and disease transmission. It is essential that the methods for insecticide monitoring in mosquito populations and interpretation of results are performed adequately, effectively and in a timely manner for improving mosquito control [10,11].

Current research on mosquito control is now focused on understanding the mosquito resistance to synthetic insecticides and developing novel strategies to overcome the resistance issues. Natural compounds that are more effective and less toxic than the synthetic ones continue to get more attention in the research community. The use of bioinsecticides, composed of botanical or plant-based compounds, has been a perfect alternative due to their minimal hazardous effects on human health and environment. In this review, we provide current knowledge on synthetic insecticides that are actively used in mosquito control and how they impact prevalence of insecticide resistance in mosquitoes. Major plant-based insecticides, their mode of action and the research about their potential mosquitocidal activity are discussed. A comprehensive understanding of how biochemical compounds can be advantageous to synthetic ones and how we can circumvent insecticide resistance issues in the fight with mosquito-borne disease transmission is provided.

2. Insecticide-Based Mosquito Control Strategies

Insecticide-based mosquito control plays an important role in efforts to reduce the transmission of mosquito-borne diseases worldwide. Two core insecticidal interventions are in use to control mosquitoes: deployment of insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS) of insecticides [10]. These interventions have been effectively used to kill mosquitoes or interfere with their host-seeking behavior to prevent disease transmission worldwide [12–20]. The global malaria cases and malaria death rates have declined about 18% and 48%, respectively, between the years 2000 and 2015, and 70% reduction in malaria cases in sub-Saharan Africa was attributed to ITNs, and 10% reduction was due to IRS [21].

Four classes of insecticides are mostly used in mosquito control programs which include pyrethroids (e.g., deltamethrin, permethrin, cypermethrin, lambda-cyhalothrin), organochlorines (e.g., DTT), organophosphates (e.g., malathion, fenitrothion), and carbamates (e.g., propoxur, bendiocarb) [10] (Figure 1). Most synthetic insecticides have physiological or behavioral impact on mosquitoes (Figure 1), and predominantly target the central nervous system of insects. Among them, pyrethroids are the most widely used insecticides for IRS and the only synthetic insecticide currently used in ITNs and fabrics, with irritant or repellent activity on mosquitoes and less mammalian toxicity [2]. They disrupt the voltage-gated sodium channels in neuronal membranes [22]. When pyrethroids bind an open channel, they prevent its closure, thus leading to a prolonged action potential

or disruption of electrical signaling in the nervous system [23–25]. This causes continuous nerve excitation and paralysis (or knockdown) of the insect and eventually its death [26].

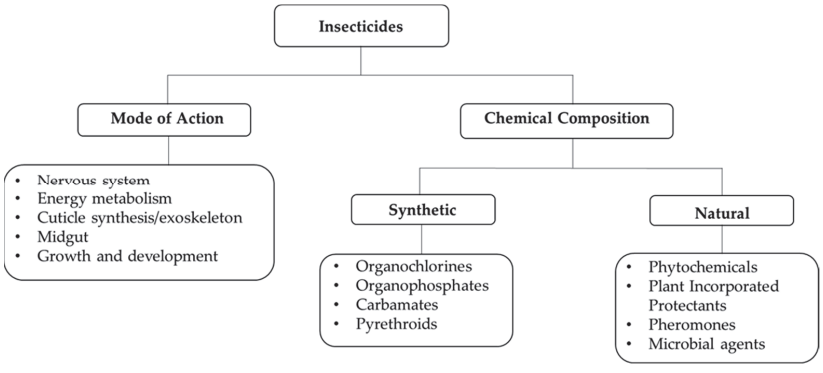

Figure 1. Classification of insecticides based on mode of action and chemical composition.

While pyrethroids have been effectively used in ITNs to control mosquitoes for a long time, prevalence of pyrethroid resistance in mosquito species causes a major problem to combat disease transmission worldwide [27–29]. Like pyrethroids, some organochlorines are also inhibitors of the insect's voltage-gated sodium channels. Dichlorodiphenyltrichloroethane (DDT) is an example that targets sodium channels, and it is the first and the most commonly used synthetic insecticide of organochlorine in residual spraying. Its low cost and high effectiveness have made it a favorable chemical for indoor wall spraying. However, resistance developed to DDT in various mosquito species and its toxic effects on humans and non-target organisms have imposed limitations or restrictions in its usage [30,31]. Other organochlorines (such as cyclodienes, dieldrin and fipronil) target γ-amino butyric acid (GABA) receptors, which are hetero-multimeric gated chloride channels in the insect's central nervous system [32]. Cyclodiene insecticides act as neurotoxicants and block the GABA receptors causing hyper-excitation of the central nervous system, convulsions, and eventually death of insects [33–35]. Organophosphates (OP) and carbamates are two other insecticides sharing similar mode of action. They inhibit acetylcholinesterase (AChE) enzyme, preventing breakdown of the neurotransmitter acetylcholine, resulting in neuromuscular overstimulation and death of insects [36–38]. Due to pyrethroid and DDT resistance issues worldwide, they have been used as alternative insecticides in IRS, but they have a shorter residual effectiveness, high toxicity to mammals, and are more costly compared to the others that limit their persistent long-term usage.

3. Insecticide Resistance in Mosquitoes

Short after its first usage in California in 1945, the resistance of mosquitoes to DDT was reported [39,40]. Since then, insecticidal resistance in mosquitoes has been reported, with a substantial increase between 2010 and 2016 [10]. In these years, insecticide resistance was found to be widespread in *Anopheles* vectors in malaria endemic African regions and insecticide resistance frequency has changed over time [10]. Understanding pyrethroid resistance development in *Anopheles* mosquitoes is particularly important because its prevalence can disable pyrethroid-treated ITN-based interventions, which are used successfully for malaria control [41,42]. Pyrethroid resistance was determined to be very high in the WHO African Region (78%), Eastern Mediterranean Region (70%), and in the South-East Asia Region (38%), Western Pacific Region (51%), but was lower in the Region of the Americas (20%). The incidence of organochlorine resistance was also similar in all WHO regions (60–70%). Carbamate resistance prevalence was between 22% and 54%, and organophosphate resistance prevalence varied widely across regions, 14% in the WHO African Region and 65%

in the WHO Western Pacific Region [10]. While resistance frequencies are generally high in most of the endemic regions, those with lower resistance frequencies could be an indication of recent gain of resistance or selection for resistant populations to insecticides [43].

Despite effective use of insecticide-based mosquito control strategies for decades, their prolonged usage is challenged by high cost, toxicity and, more importantly, the development of resistance to the synthetic insecticides. Insecticide resistance is mostly inferred to the ability of insects to survive exposure to a standard dose of insecticide, owing to physiological or behavioral adaptation [44]. Resistance can be developed due to misusage or overdose usage of insecticides and selection pressure on the insect populations [45]. The question "when does the resistance emerge?" depends on the mechanism of resistance, known susceptibility, cost effectiveness and availability [45]. Various resistance mechanisms have been observed in mosquitoes: changes in their metabolism (changes in enzymes leading due rapid detoxification of insecticides), alterations in target-sites (prevention of insecticides to their target sites), penetration resistance (cuticle barrier diminishes insecticide penetration) and behavioral resistance (changes in their response to insecticidal effect) [46–49]. These mechanisms can be determined by using bioassays, biochemical assays, and molecular techniques through assessment of resistance alleles, analyzing whether metabolic enzymes are upregulated, or determination of the percent mortality rate upon exposure to a given insecticide.

In mosquitoes, alterations of target site nerve receptors (e.g., mutations in *kdr*, *Rdl* and *Ace-1R* genes) and detoxification due to increased or modified enzyme activities (e.g., monooxygenases (P450s), glutathione-S-transferases and carboxylesterases) are the two major mechanisms responsible for insecticide resistance. According to the insecticide resistance monitoring data for 2010 to 2016, almost 70% of the assays to test resistance mechanisms included detection of the presence or absence of target-site mutations and their frequencies in WHO regions [10]. Target site alterations in mosquitoes involve knockdown resistance (*kdr*) mutations (L1014F or L1014S) in the voltage-gated sodium channel gene which causes inability of the insecticides to bind their cognate receptors [50–55]. Occurrence of *kdr* mutations causes insensitivity to pyrethroids and DDT [56,57]. A *kdr*-resistant strain of *An. gambiae* has shown to be less affected by pyrethroids than the susceptible strain [58]. In the last few decades, *kdr* resistance mutations in different mosquito populations have expanded significantly which restricts pyrethroid usage in mosquito control [59]. Another target-site mutation, the AChE gene mutation (*Ace-1R*), causes resistance to organophosphates and carbamates. In mosquitoes, a G119S mutation in the *Ace-1R* gene encoding AChE causes resistance to organophosphate and carbamate insecticides and the mutation frequency is increasing in natural mosquito populations [60–63]. A substitution mutation of alanine-to-serine/glycine (A296S/G) mutation, *Rdl*, in the second transmembrane domain of the GABA receptor subunit causes resistance to organochlorine insecticides and insensitivity in mosquitoes [35,64–69].

Mosquitoes have metabolic enzymes, mainly "detoxifying enzymes" that are responsible for biodegradation of insecticides and elimination of their insecticidal effects. Upon exposure to synthetic insecticides, detoxifying enzyme activity increases (due to increased gene amplification or upregulation) which result in insecticide-resistant mosquitoes [46]. Three classes of detoxifying enzymes are involved in insecticide-resistance in mosquitoes: cytochrome P450 monooxygenases (CYP), glutathione-S-transferases (GST) and carboxylcholinesterases (CCE) associated with pyrethroid, organochloride, and OP and carbamate resistances, respectively. Cytochrome P450 enzymes are involved in the metabolism of all four classes of insecticides. It is found that elevated levels of P450 activity resulted in pyrethroid resistant mosquito vectors [70–74]. Several CYPs are identified in mosquitoes and CYP overexpression is reported from insecticide resistant mosquito populations [45,59,75–77]. Knockdown of the CYP through the RNA-interference technique also showed that mosquitoes become sensitive to pyrethroids [78–80]. Glutathione S-transferases comprise a diverse family of enzymes involved in detoxification of insecticides (e.g., pyrethroids and DTT) in mosquitoes [81]. An increase in the gene expression levels of various GSTs has been

detected in DDT-resistant and pyrethroid-resistant mosquitoes [82–88]. Additionally, a GST gene silencing study indicated an increase in the susceptibility to pyrethroid insecticide which shows that GSTs are involved in insecticide-resistance in mosquitoes [86]. Increased esterase detoxification in OP resistance has been studied most extensively in *Culex* mosquitoes [72,89]. These enzymes sequester the insecticide and interfere with its association with the target AChE by rapid binding and slow turning over of the insecticide [90]. The increase in the activity of esterases was due to overproduction of the enzymes, resulting from co-amplification of two esterase genes, *estα2* and *estβ2*, in OP-resistant individuals [91,92].

It is evident that cross-resistance causes major issues in the management of insecticide resistance through the approaches discussed above. These mechanisms can cause resistance to more than one class of insecticide (with similar mode of action) due to prolonged and intensive usage of these chemicals. For example, *Culex* mosquitoes that are resistant to a pyrethroid insecticide also show resistance to OP and other insecticides [93,94]. Pyrethroid-resistant *Anopheline* mosquitoes also show resistance to OPs due to constitutively elevated P450 levels leading to cross-resistance [95]. Moreover, insecticide resistance is genetically mediated and can be fixed in mosquito populations in such that individuals with the resistance gene will probably have a selective advantage in the presence of the insecticide [96,97]. Furthermore, mosquitoes that survive insecticide exposures possibly have the chance of passing those traits to their offspring which causes an increase in the percentage of resistant individuals in the next generations in those populations [48]. If resistance gene frequency increases in the populations, this can cause more resistant individuals to circumvent insecticidal exposures. Taken together, the emergence and spread of insecticide resistance, cross-resistance, and increased resistance gene frequencies in mosquito populations significantly effects mosquito-borne disease control and elimination and highlights the need for alternative strategies. There has been a great interest for safe and healthy biological control strategies and development of novel interventions to overcome problems associated with synthetic insecticides. Hence, extensive research for another class of insecticide for mosquito control, named "bioinsecticide", is an ongoing process and novel natural compounds are being investigated to replace conventional synthetic insecticides. In this review, we will focus on plant-based bioinsecticides with potential activity in mosquito control.

4. Plant-Based Bioinsecticides

Bioinsecticides are derived from natural products, such as bioactive compounds of plants, pheromones, and from microorganisms, such as bacteria, fungi, virus, or protozoan. There are four major classes of bioinsecticides based on their nature of origin: phytochemicals, microbial pesticides, plant-incorporated protectants (PIPs), and pheromones [98] (Figure 1). They have been effectively used in pest management and generation of sustainable agricultural products [99,100]. They are less toxic, target-specific, highly effective in small quantities and biodegradable, which makes them excellent alternatives to synthetic compounds. More importantly, mosquitoes are developing resistance to synthetic compounds, a burden that needs to be resolved for successful mosquito disease control. Since biopesticides induce less insect resistance [101,102], most studies now focus on discovery of candidate natural compounds with potential effects on mosquitoes to combat mosquito-borne disease transmission.

Plants have evolved to develop many defensive chemical compounds against pathogenic microorganisms and insects. These biologically active chemical compounds, referred to as "phytochemicals", function as repellents, toxins, feeding deterrents, and growth regulators against insects [103]. Various parts of higher plants (leaves, roots, stems, seeds, barks, fruits, peels of fruit and resin), the whole body of little herbs, or mixture of different plants can be used for an effective plant-based insecticide. The activity of a phytochemical can change significantly depending on the plant species, plant part and its age, polarity of solvents used during extraction procedures and mosquito species [104]. Phytochemicals show

their effects through targeting important cell components and affecting insect physiology in different ways; via inhibition of AChE and GABA-gated chloride channel activity, disruption of sodium-potassium ion exchange and nerve cell membrane action, blocking calcium channels, and activation of nicotinic acetylcholine receptors and octopamine receptors [105]. Moreover, phytochemicals can cause cellular destruction of epithelial cells in the midgut of mosquitoes and affect metamorphosis [106,107].

Several phytochemicals have been reported for their mosquitocidal activities [104,108]. These chemical compounds are mostly secondary metabolites, such as essential oils, alkaloids, phenols, terpenoids, steroids, and phenolics from different plants. Phytochemicals in plant species are diverse and discovery of those with mosquitocidal activities, which are governed by changes in expression levels of detoxifying enzymes, are of great importance to control mosquitoes. In the following sections, we provide the current knowledge on mosquitocidal plant-based compounds and their activities for a better understanding of their efficacy to prevent mosquito-borne diseases.

5. Plant-Based Compounds and Mosquito Control

Plant-based compounds possess larvicidal, ovicidal and repellent activities on early or adult stages of mosquitoes, affecting nervous, respiratory, endocrine, and water balance systems. Ovicidal and larvicidal effects of many plant compounds have been extensively studied since mosquitoes are immobile at these stages and they can be efficiently eliminated before they emerge as adults. Repellent compounds are effective in keeping human hosts from mosquito bites for a blood-meal. Thus, understanding the mosquito olfactory system is vital for determination of repellent compounds. Insect repellents affect the olfactory receptor neurons via modifying or blocking its response, which in turn, elicit avoidance behavior or a change in the host-seeking behavior of mosquitoes [109,110]. There are many plant compounds with repellent activities. Essential oils, alkaloids, and aromatic compounds from various plants are commonly used for plant-based mosquito repellents [111] and they have shown to interfere with the mosquito host-seeking behavior when applied on human skin or used as indoor spraying [112]. Insecticidal and repellent activities of four major plant metabolites (essential oils, neem, pyrethrum, alkaloids) and other plant compounds (flavonoids and rotenone) are discussed in detail (Table 1).

5.1. Essential Oils

Essential oils have been efficiently used against a variety of pests and for crop protection in the world and they are potential alternatives to synthetic insecticides used against mosquitoes. Essential oils are very complex natural mixtures that consist of a variety of volatile molecules, which are hydrocarbons (terpenes and sesquiterpenes), oxygenated hydrocarbons and phenylpropenes (Table 1). Essential oils are synthesized in the cytoplasm and plastids of plant cells through mevalonic acid and 2-*C*-methyl-erythritol 4-phosphate (MEP) pathways, respectively [113]. Essential oils target the insect nervous system and cause neurotoxic effects through several mechanisms by inhibiting the activity of AChE, and blocking octopamine receptors and GABA-gated chloride channels [114,115]. About 90% of essential oils are composed of monoterpenes, which are determined to be active ingredients for potential plant-based larvicides and cause inhibition of AChE activity in insects [116]. Monoterpenes, such as linalool, cuminaldehyde, 1,8-cineole, limonene and fenchone, cause inhibition of AChE and accumulation of acetylcholine in synapses and state of permanent stimulation, which results in ataxia [117,118]. According to Hideyukiu and Mitsuo [119], a mixture of monoterpenoids is a more potent inhibitor of AChE than single monoterpenoid application and acts synergistically.

Table 1. An overview of insecticidal activity and mechanism of action of various plant-based compounds against mosquito species.

Type of Botanical Product	Plant Family	Activity	Mechanism of Action	Mosquito Species	References
Essential Oils Monoterpenes: linalool, cuminaldehyde, 1,8-cineole, limonene, fenchone, eugenol, γ-terpineol, cinnamic alcohol, geraniol, β-citronellol, *P*-menthane-3,8 diol, α-pinene, β-pinene, *p*-cymene, thymol, terpinolene, camphor, citronellal, sabinene, carvacrol Sesquiterpenes: guaiol, α-bisabolol, α-cadinol, germacrene D, β-caryophyllene, nootkatone Diterpenoids: diterpene alcohol, phytol Aromatic phenol Coumarin	Anacardiaceae Annonaceae Apiaceae Asteraceae Geraniaceae Lamiaceae Lauraceae Poaceae Rutaceae Myrtaceae Verbenaceae	larvicidal, pupaecidal, ovicidal, adulticidal, repellent, antifeedant, growth and reproduction inhibitors	Inhibition of AChE Blockage of GABA-gated chloride channels Agonist of octopamine receptors	*Cx. pipiens pallens* *Cx. quinquefasciatus* *Cx. pipiens biotype molestus* *Ae. aegypti* *Ae. albopictus* *An. gambiae* *An. stephensi*	[120–146]
Neem oil azadirachtin, meliantriol, salannin, desacetyl salannin, nimbin, desacetyl nimbin, nimbidin, nimbolide, deacetylgedunin, gedunin, 17-hydroxyazadiradione, deacetylnimbin	Meliaceae	repellent, ovicidal, larvicidal, feeding deterrence, fecundity suppression, toxicity, growth regulation, oviposition deterrence	growth inhibitors, hormonal disruption (ecdysone blocker), molting aberrations, interference with phagostimulants	*An. gambiae* *Ae. aegypti* *Ae. albopictus* *An. stephensi* *Cx. quinquefasciatus*	[147–165]

Table 1. *Cont.*

Type of Botanical Product	Plant Family	Activity	Mechanism of Action	Mosquito Species	References
Pyrethrum esters of chrysanthemic acid: pyrethrin I, cinerin I, jasmolin I esters of pyrethric acid: pyrethrin II, cinerin II, jasmolin II	Asteraceae	repellent, knock-down effect, blood-feding inhibition	voltage-gated sodium channel modulator	*An. gambiae*	[166–170]
Alkaloids alpha-solanin ricinine pyridine nicotine diterpene nornicotine anabasine	Berberidaceae Fabaceae Solanaceae Ranunculaceae Euphorbiaceae	repellent, larvicidal	interfering with cellular and physiological functions, inhibition of AChE activity, regulation of hormone activity, toxicity, agonist of acetycholine receptor	*Ae. aegypti* *An. arabiensis* *An. gambiae* *Ae. albopictus* *An. stephensi* *Cx. pipiens*	[171–181]
Flavonoids	Zingiberaceae	larvicidal	inhibition of AChE, degradation of cell membranes acting as stomach poisons	*Ae. aegypti*	[182–184]
Rotenone	Fabaceae	larvicidal	inhibitor of the cellular respiration system	*Ae. aegypti*	[185]

The octopaminergic system of insects is another target for essential oils that block octopamine receptors and cause acute and sub-lethal behavioral effects on insects. The increase in cyclic AMP levels, induced upon binding of octopamine to octopamine-receptors, can be inhibited by a mixture of essential oils (eugenol, γ-terpineol and cinnamic alcohol). Moreover, octopamine receptor binding is significantly reduced with low doses of eugenol alone [120,121]. Another possible target for essential oils is ligand-gated chloride channels. Essential oils consist of monoterpenes, such as linalool, methyl eugenol, estragole, citronellal, inhibit GABA-gated chloride channels by binding at the receptor site and increase the chloride anion influx into the neurons, which lead to hyper-excitation of the central nervous system, convulsions, and finally death of insects [122,123].

Many plant oils possess ovicidal, larvicidal, pupaecidal and repellent activities against various mosquito species, some of which will be discussed below. Essential oils of plants from the Lamiaceae, Poaceae, Rutaceae and Myrtaceae families are well-known for repellent activity [103]. Essential oils obtained from citronella, lemon and eucalyptus are commercially available and recommended by the U.S. Environmental Protection Agency (US EPA) as repellent ingredients for application on the skin because of their low toxicity. For example, *P*-menthane-3,8 diol (PMD) is an active component of the lemon eucalyptus plant and responsible for the repellency in mosquitoes [124].

Most of the monoterpenes and sesquiterpenes of essential oils are known with repellent activities [125]. Among monoterpenes, α-pinene, γ-pinene, *p*-cymene, eugenol, limonene, thymol, terpinolene, citronellol, camphor and citronellal are responsible for mosquito repellency [126,127]. Representative molecules of sesquiterpenes are guaiol, α-bisabolol, α-cadinol, germacrene D, β-caryophyllene and nootkatone. β-caryophyllene is known to exhibit strong repellent activity against *Aedes* mosquitoes [126]. Repellent and larvicidal activities of monoterpenes from the essential oils of *Thymus* plant against *Cx. pipiens pallens*, *Cx. quinquefasciatus*, and *Cx. pipiens* biotype *molestus* have been determined [128–130]. Larvicidal activities of phenolic terpenes, such as thymol and carvacrol, of *Satureja* species were observed against *Cx. pipiens* biotype *molestus* [131]. Moreover, repellent and larvicidal activities of carvacrol were determined in the field trials against *Ae. albopictus* mosquitoes in Bologna (Italy) [132]. *Cinnamomum osmophloeum* and *Carum copticum* essential oils had larvicidal activity against *Cx. quinquefasciatus* and *Cx. pipiens*, respectively [107,133]. Toxicity of β-citronellol, geraniol and linalool from *Pelargonium roseum* essential oil was also detected in *Cx. pipiens* [134]. High larvicidal and pupaecidal activities of essential oils from *Cinnamomum verum*, *Citrus aurantifolia*, *Cuminum cyminum*, *Syzygium aromaticum*, *Laurus nobilis*, *Lippia berlandieri* and *Pimpinella anisum* were reported from *Cx. quinquefasciatus* [135]. *Artemisia absinthium* essential oils also showed toxic effects against larval populations of *Aedes*, *Anopheles*, and *Culex* mosquitoes [136]. Essential oils isolated from *Tagetes lucida*, *Lippia alba*, *Lippia origanoides*, *Eucalyptus citriodora*, *Cymbopogon citratus*, *Cymbopogon flexuosus*, *Citrus sinensis*, *Swinglea glutinosa*, and *Cananga odorata* plants showed larvicidal activities on *Ae. aegypti* larvae [137]. Oviposition deterrence and ovicidal activity of some of essential oils, peppermint oil, basil oil, rosemary oil, and citronella oil from *Mentha piperita*, *Ocimum basilicum*, *Rosmarinus officinalis*, *Cymbopogon nardus* and *Apium graveolens* were also reported in *Ae. aegypti* [138]. Manh et al. [139] also showed toxicity of essential oils from *Eucalyptus* and *Cymbopogon* aromatic plants to the larvae of *Ae. aegypti*. Essential oils also cause toxicity at different developmental stages and have repellent activities against adult *Anopheles* mosquitoes [140]. Essential oils extracted from *Cymbopogon proximus*, *Lippia multiflora* and *Ocimum canum* had larvicidal and ovicidal activities against *An. gambiae* and *Ae. aegypti* mosquitoes [141]. Besides monoterpenes and sesquiterpenes, phytol (a diterpene alcohol) and coumarin (an aromatic phenol) were both determined to be responsible for the biting deterrence effect in *Ae. aegypti* [142].

Repellent activity of essential oils is generally attributed to individual chemical compounds, but synergistic effects of plant metabolites have been observed when the effect of an active compound is enhanced by other major compounds or modulated by minor compounds. The efficacy of the major compounds is enhanced by minor compounds

through different mechanisms, which may cause higher bioreactivity compared to isolated compounds of essential oils. The synergistic effect is also observed with mixture of oils. The synergistic action of the major compounds in essential oils results in higher repellent and larvicidal activity and toxicity to insects [140,143–145]. A combination of blends assayed on *An. gambiae* mosquitoes indicated that blends of oils showed higher repellency compared to the individual oil used [146]. It has been also reported that essential oils composed of a mixture of active components might reduce resistance in mosquito population by acting at different target sites or with a different mode of action [139].

5.2. Neem

Neem-based insecticides are extensively used for protection against various pests all over the world. Neem trees, *Azadirachta indica*, is a member of the Meliaceae family and are originated from India and distributed throughout all South- and Southeast-Asian countries, including Pakistan, Sri Lanka, Thailand, Malaysia, and Indonesia [147]. The main product of the neem is the oil extracted from the seeds and contains at least 100 active compounds, including azadirachtin, meliantriol, salannin, desacetyl salannin, nimbin, desacetyl nimbin, nimbidin and nimbolides [148]. Limonoids are the major active compound of the neem oil and act as an insect growth inhibitor. Azadirachtin is a triterpenoid and highly oxidized limonoid, one of the most potent active compounds of the neem extract and found in higher concentrations (0.2–0.6%) in the seeds of the neem compared to other parts of the neem tree [149,150]. Various isomers of azadirachtin (azadirachtin A to G) were identified and azadirachtin A and B isomers are the most abundant isomers in the plant tissues. In addition, azadirachtin A is the most active biological ingredient which shows insecticidal activity compared to the other analogs [151–153].

Generally, neem-based products are effective in the juvenile stages of insects. Azadirachtin is structurally similar to insect hormones known as ecdysones that are involved in the process of metamorphosis. The main mechanism of action of azadirachtin is to impair the homeostasis of insect hormones by interfering with the endocrine system. Azadirachtin acts as ecdysone blocker and causes severe growth and molting aberrations by affecting ecdysteroid and juvenile hormone titers [154]. The feeding deterrent activity of azadirachtin is mediated through azadirachtin's interference with phagostimulants that are important in normal feeding behavior of mosquitos [155].

Neem-based biopesticides have a wide range of effects against insects, such as repellency, feeding deterrence, ovicidal activity, fecundity suppression, toxicity, insect growth regulation, deterrence of egg-laying, disruption of growth and reproduction, and inhibition of metamorphosis [156–160]. Larvicidal activity of the neem oil has been reported in controlling mosquito larvae in different breeding sites under natural field conditions [161]. Ayinde et al. [162] reported the repellent and larvicidal potential of the emulsified neem seed oil formulation as a suitable alternative for commercially available insecticides against *An. gambiae* in Nigeria. Oils of neem and karanj were also found to have larvicidal, ovicidal and oviposition deterrent activities against *Ae. aegypti* and *Ae. albopictus* mosquitoes [163]. The effects of the neem limonoids azadirachtin, salannin, deacetylgedunin, gedunin, 17-hydroxyazadiradione and deacetylnimbin were analyzed, and azadirachtin, salannin and deacetylgedunin showed the highest larvicidal activity against *An. stephensi* [164]. Larval mortality and repellent activity were also achieved from neem essential oils against *An. gambiae* [162]. A neem extract, neemarin, also showed significant mortality rates at larvae, pupae, and adult stages of *Cx. quinquefasciatus* and *An. stephensi*, where the former showed lower mortality rates [165].

5.3. Pyrethrum

Pyrethrum is a plant-based insecticide obtained from flower heads of *Tanacetum cinerariifolium*. Pyrethrum extract is composed of six active ingredients derived from esters of chrysanthemic acid: pyrethrin I, cinerin I, and jasmolin I, and esters of pyrethric acid: pyrethrin II, cinerin II, and jasmolin II [166]. They target the nervous system of insects

and cause neurotoxic effects through blocking the voltage-gated sodium channels in nerve axons, thereby cause hyperactivity and convulsions by a rapid knockdown effect [167]. The mode of action of pyrethrins is similar to that of DDT and many synthetic organochlorine insecticides. Thus, pyrethrins can be alternatively used instead of organophosphates and organochlorides. While it is less toxic to mammals, it has higher toxicity to fish and aquatic invertebrates. When used together with a conventional synergist, such as piperonyl butoxide (PBO), their activity is increased and harmful effects to non-target organisms are reduced [168]. The usage of natural pyrethrins in mosquito control is supported with the finding that pyrethrum had knock-down effect, repellency, and blood-feeding inhibition in pyrethroid-resistant *An. gambiae* strains [169]. Electroantennogram responses of pyrethrum in *Ae. aegypti* and *An. gambiae* mosquitoes were detected while no response is observed in maxillary palps, indicating that the repellency effect of pyrethrum is mediated by the olfactory systems of mosquitoes [170]. Moreover, the molecular mechanism of pyrethrum repellency was investigated and a synergistic mechanism involving dual activation of olfactory repellency pathways and voltage-gated sodium channels has been determined [170].

5.4. Alkaloids

Alkaloids are nitrogen-containing natural products found in bacteria, fungi, animals, and plants. They are commonly isolated from plants and found in large quantities in many members of the Berberidaceae, Fabaceae, Solanaceae, and Ranunculaceae families. The alkaloids obtained from these plants are used extensively in conventional insect repellents [171–173]. The mode of action of alkaloids varies depending on the type of alkaloids and interferes with major cellular and physiological functions by affecting AChE receptors in the nervous system, regulating hormonal activity, and causing toxicity [174]. Alkaloids are not volatile like essential oils. However, they could be used as repellents against mosquitoes by burning plants to generate an insecticidal smoke that repels insects and directly causes toxicity [124]. In *Ae. aegypti*, the inhibitory effect of natural alkaloids on AChE activity was determined by using molecular docking studies. Among the 25 different alkaloids tested, alpha-solanine has been found to fit into the AChE1 binding pocket and potentially be the best inhibitor of AChE1 [175].

Extracts of the castor bean (*Ricinus communis*, Euphorbiaceae) contain the alkaloid ricinine and have a strong insecticidal effect. It showed strong larvicidal activity against larvae of *An. arabiensis* [176]. Additionally, pyridine alkaloid from *R. communis* showed bioactivity against *An. gambiae* larvae and adults [177]. The larvicidal activity of alkaloids against *Ae. albopictus*, *Cx. pipiens pallens* and *Ae. aegypti* has also been determined [178,179]. Alkaloid from *Arachis hypogaea* plant also had larvicidal toxicity against *An. stephensi* and *Ae. aegypti* mosquitoes [180].

Nicotine is an alkaloid derived from tobacco plant (*Nicotiana tobacco*) that mostly consists of phenolic compounds, such as nicotine and diterpene. Nicotine, nornicotine and anabasine mimic the neurotransmitter acetylcholine, which causes symptoms similar to organophosphate or carbamate insecticides [160]. Extracts of tobacco leaves were mixed with bio-oil and high repellent activity was observed against *Ae. aegypti* [181]. Furthermore, nicotine has been found to be the most dominant compound among the other active compounds of the repellent mixture, including nicotine, d-limonene, indole, and pyridine. In addition, the repellent compound was harmless to human skin as confirmed by sensitivity tests on volunteers.

5.5. Other Plant Compounds

Besides the most common plant-based bioinsecticides mentioned above, there are other natural plant metabolites that show insecticidal properties. Among them, flavonoids elicit larvicidal activity by inhibiting AChE in mosquito larvae [182]. They could also act as respiratory inhibitors and result in the disturbance of the larval respiratory system. Alkaloids have multiple effects including inhibition of the AChE enzyme, degradation

of cell membranes, and they may act as stomach poisons [182]. It has been shown that flavonoid and alkaloid components of bangle rhizome extract from *Zingiber montanum* act differently against *Ae. aegypti* [183]. Flavonoids from *Derris trifoliata* extract also exhibited larvicidal activity against *Ae. aegypti* [184]. Rotenone is an isoflavonoid extracted from roots and stems of *Derris* (*Derris elliptica*, *Derris involute*), *Lonchocarpus* (*Lonchocarpus utilis*, *Lonchocarpus urucu*) and *Tephrosia virginiana* [160]. It has long been used as a biopesticide due to less harmful effects to the environment. Rotenone has the potential to be used as a larvicide to control mosquitoes and interferes with the cellular respiration system of insects and prevents energy production [185].

6. Assessment of Plant-Based Bioinsecticide Efficacy in Mosquito Control

It is important that inherent activity of candidate bioinsecticides should be assessed before they can be effectively used against mosquito populations. The World Health Organization has established methods to screen the efficacy and field application acceptability of new compounds as potential mosquito larvicides and adulticides (for IRS and ITNs); they are laboratory studies, small-scale and large-scale field trials [186–188]. Laboratory studies focus on determination of biopotency, efficacy, residual activity, irritant or repellent properties, diagnostic concentration, and possible cross-resistance of candidate larvicides or adulticides. In laboratory bioassays, mosquito larvae are exposed to various concentrations of larvicides, and a mortality rate based on lethal concentration (LC) of the larvicide for 50% and 90% mortality (LC50 and LC90) or for 50% and 90% inhibition of adult emergence (IE50 and IE90) is recorded. LC values are determined and can then be compared with the LC50 or LC90 values of other insecticides to assess the activity of the compound as "sufficiently effective". For adulticides, LC is determined by tarsal contact to treated papers. The "time to first take-off" (FT) for the 50% and 90% of the mosquitoes to take off (FT50 and FT90) after exposure to treated substrates are measured to determine the irritant or repellent activity of an adulticide. Insecticide-treated nets are used for bioassays of adult mosquitoes to determine the efficacy and residual activity of different dosages of the candidate compounds. Moreover, efficacy and wash-resistance of ITNs against susceptible mosquito species should be determined using standard WHO cone bioassays or tunnel tests [188]. The efficacy criteria for cone bioassays are $\geq$80% mortality or $\geq$95% knock-down, and for the tunnel test, it is $\geq$80% mortality or $\geq$90% blood-feeding inhibition. Candidate larvicides and adulticides are also tested against multi-resistant mosquito strains and a susceptible reference strain to assess the cross-resistance and, if detected, biochemical, immunological, and molecular methods are used to determine the mechanism of resistance [189].

Once candidate compounds are selected from laboratory tests, they are subjected to small-scale field testing in natural breeding sites (such as drains sewage water tanks, ponds, rice plots, etc.) or under simulated field conditions (artificial containers filled with water, experimental huts). Larvicidal efficacy is determined by the level of inhibition of emergence of adults and the percentage reduction in larval and pupal densities, while adulticidal efficacy can be assessed in terms of mortality, residual effect, deterrence, blood-feeding inhibition and induced exophily. These trials elucidate efficacy of candidate compounds against different mosquito species in different breeding sites, determine optimum field application dosage of the compound and possible impact on the mosquito behavior. Abiotic parameters that may influence the efficacy of the product and effect on non-target organisms can also be observed. Those larvicides and adulticides that show promise in small-scale field trials should be validated in larger-scale field trials against natural mosquito populations in natural breeding habitats using optimum field dosages. At this stage, the storage, handling, and application of the insecticide formulation should be considered for proper functioning of application and dispersal of the bioinsecticide in natural ecosystems.

There are also potential limitations to the efficacy of bioinsecticides, such as environmental conditions, mosquito fitness, mosquito resistance as well as the parts of the plants used, solvents used in extraction steps, insecticide dose and exposure time [190,191]. These effects should be considered for successful assessment of novel bioinsecticides in mosquito

control. While efficacy tests provide promising information on possible mosquitocidal effects, new compounds from plant origin, the identification of actual active ingredient for efficacy and their mode of action are still waiting to be resolved.

7. Effective Use of Plant-Based Bioinsecticides in Resistant Mosquito Populations

Most of the bioinsecticides are now effective alternatives to chemical insecticides and have become an integral part of the integrated mosquito management (IMM) programs because the development of resistance to bioinsecticides is low due to their multiple mode of actions [192,193]. The synergic mixture of the active compounds in plant extracts also minimizes resistance development [167]. However, resistance already developed to extensively used chemical insecticides is a major problem that limits the success rate of novel bioinsecticides against mosquito populations. Insecticide resistance should be reduced or reverted (which takes time) in order to apply new and effective bioinsecticides in resistant populations. Surveillance of mosquito resistance and effective resistance management strategies should be routinely conducted to determine the levels, mechanisms, and geographic distribution of resistance in field populations of mosquitoes for increasing efficacy of bioinsecticides [44]. Moreover, proper application technologies should be considered as they greatly influence the bioinsecticide efficacy.

Surveillance of resistance development to many different insecticides are determined by dose-mortality bioassays, the World Health Organization tube testing, and Centers for Disease Control and Prevention (CDC) bottle bioassay for mosquitoes [11,44,194,195]. In the dose-mortality assay, the resistance ratio (RR) is determined in a susceptible population to monitor changes in resistance over time. The RR is calculated from LC50 values of the field and susceptible populations, in which an RR lower than five indicates susceptibility or low resistance and an RR value higher than ten indicates high resistance. In the WHO tube testing, the insecticide susceptibility status of the selected mosquitoes is evaluated through susceptibility tests measuring the mortality rate twenty-four hour after exposure [44]. A mortality rate lower than 98% indicates occurrence of resistance and should be confirmed with biochemical and molecular analysis. A mortality rate less than 90% confirms the existence of resistant genes in the tested mosquito populations. The CDC bottle bioassay is a measure of insecticide effectiveness, where diagnostic doses (DDs) and diagnostic times (DTs) are determined for candidate compounds using susceptible mosquitoes prior to testing in field mosquito populations. The DD is a measure of insecticide dose that kills 100% of susceptible mosquitoes within a certain period of time (DT). A mortality rate lower than 97% is an indication of resistance that needs to be confirmed, and below 80% suggests strong resistance at the recommended DT. The DD and DT values for some active ingredients are available for *Anopheles* and *Aedes* mosquito populations and these parameters should be defined for a particular insecticide and mosquito population [195].

It is evident that no single strategy is effective enough to solve insecticide resistance of mosquitoes. According to the WHO [44], one strategy to prevent the resistance problem is rotational usage of different classes of bioinsecticides with different modes of action. There are several new plant-based larvicides with different modes of action (discussed in Section 5) and they could be good alternatives for mosquito control in larval stages. Additionally, multiple interventions that affect different stages of mosquitoes (such as larvae and adults) can be used together to manage insecticide resistance. It is also suggested that different classes of insecticides with different modes of action can be used in neighboring geographic locations. To successfully implement these strategies, knowledge of the mode of action of the novel bioinsecticide is essential. The resistance mechanism developed by the local population of mosquitoes should also be determined to reduce cross-resistance effects.

RNA interference (RNAi) mediated loss-of-function technique has been proposed for pest management programs [196,197] and to study insecticide resistance [198]. Genes responsible for resistance development in insects (e.g., genes for DDT or pyrethroid resistance) can be identified and used as a target for the development of novel RNAi based insecticides. Several delivery methods including nonmicrobial and microbial are used

routinely to induce RNAi in mosquito larvae [199]. Nonmicrobial delivery methods consist of soaking, injection, nanoparticles and dehydration and rehydration. Although soaking and injection methods are used as excellent tools in RNAi research, they have no application in the field. Soaking, injection methods and nanoparticles have been effectively used to introduce dsRNA into first-instar *Ae. aegypti* larvae [200] and fourth instars of *Ae. aegypti* [201]. In mosquitoes, a chitosan/dsRNA-based nanoparticle has also been used in feeding the larvae of *An. gambiae* mosquitoes which led to successful gene silencing of two chitin synthase genes and increased susceptibilities to DTT [202]. Such an RNAi-based bioinsecticide can be potentially used as an effective strategy to enhance the efficacy of new bioinsecticides for mosquito control.

Another technology used for the manipulation of insect behavior is "Specialized Pheromone and Lure Application Technology (SPLAT)". SPLAT is a chemical controlled-release emulsion technology, and it has been used as an alternative management strategy to target the aquatic life stages of mosquitoes [203]. SPLAT emulsions can be formulated by using a variety of compounds, such as sex pheromones, attractants, repellents, phagostimulants and insecticides. SPLAT consists of both aqueous and non-aqueous components. The aqueous component of the SPLAT emulsion is involved in the liquid property of the product and evaporates within 3 h upon application. The non-aqueous component of the emulsion is the controlled-release device that releases active ingredients (e.g., semiochemical or pesticides) at a controlled rate for 2 weeks to 6 months by protecting the active ingredients from environmental, chemical, and biological degradation. It has been reported that combination of attractant and larvicidal agents in a single formulation and biodegradable matrices causes significant increase in larval mosquito mortality, specifically *Cx. quinquefasciatus*, compared to formulations consisting of larvicidal agents alone in semi-field trials (e.g., large-screened greenhouse and emulating field conditions) [204]. The major benefits of this technology are a timely-manner release of both pheromone and insecticide, reduced insecticide resistance, and persistence in the field [203].

8. How to Improve Plant-Based Bioinsecticide Efficacy in Mosquito Control Strategies?

Synthetic chemicals used to control mosquitoes are now causing serious health problems and, more importantly, resistant mosquitoes that lead to search for more effective, healthier, safer, and eco-friendly natural solutions. Phytochemicals derived from plant resources are excellent targets to search for bioactive compounds because plants synthesize these chemicals naturally in response to their environment (such as against insect predators and microbial attacks), thus, plants are indeed natural insecticide sources. While searching the literature for plant-based compounds, we have encountered a tremendous number of efforts to identify and evaluate compounds that could have potential mosquitocidal activity with negative impact on mosquito physiology at different development stages. Since phytochemicals have multiple modes of action and exert their effects on multiple target sites in insects, their efficacy can be enhanced when used as a blend (e.g., mixture of oils) against mosquitoes. In addition, insects are more likely to develop resistance to a single chemical compound rather than a mixture of compounds. Thus, a combinatorial usage of phytochemicals would limit development of resistance in mosquitoes. Phytochemicals have short residual half-life which could be advantageous when synergistically used together with other biological control agents [205]. It is encouraging that these features of phytochemicals make them alternative natural solutions for the development of suitable products to interfere with the mosquito–host interaction and reduce disease transmission.

Among the phytochemicals, essential oils are extensively studied and their repellent activities against mosquitoes makes them favorable natural chemicals. However, they are volatile compounds, and this causes issues in their long-term applications in mosquito control. In recent years, new technologies, such as microencapsulation and nanoemulsion, have been used to overcome this problem by enhancing the duration and efficacy of essential oils [140]. Since ITNs are one of the major intervention methods to control

mosquitoes, the incorporation of plant-based insect repellents in fabrics seems a prompt and alternative way to provide safer protection against mosquito bites. Fabrics treated with microencapsulated citronella essential oil have been reported to provide higher repellent activity and longer lasting protection, up to three weeks, against insects compared to the fabrics sprayed with ethanol solution of the essential oil [206]. Grancaric et al. [207] also reported that microencapsulated immortelle oil had the highest repellent efficacy against *Ae. aegypti* compared to immortelle oil alone on cotton samples. In another study, microcapsules composed of two biopesticides, namely citronella essential oil and citriodiol, were prepared and applied to cotton textiles using a variety of techniques. As a result, citriodiol-treated cotton fabrics had a prolonged durability, and 100% repellent activity for more than 30 days after its application [208]. Additionally, encapsulation of citronella oil into microcapsules of poly ε-caprolactone has been considered as an effective and sustained release system with potential application in protection against mosquitoes [209]. Encapsulated citronella oil nanoemulsions prepared by high pressure homogenization at varying amounts of surfactant and glycerol were tested for mosquito repellency. It has been shown that increasing concentration of glycerol and surfactant improved the stability of the emulsion causing prolonged mosquito protection [210]. These results clearly indicate that through microencapsulation and nanoemulsion formulation technologies, effective and longer usage of essential oils on cotton fabrics or ITNs can be achieved.

Neem-based insecticides can also be effectively used for the control of mosquitoes. They are considered more eco-friendly than synthetic insecticides and are less prone to induce resistance because of their multiple modes of action on insects. Another advantage of neem oil formulations is that it causes mortality at relatively low concentrations making them potential alternatives to synthetic insecticides in the control of malaria vectors. Microencapsulation of neem seed oil and karanja oil has been used for the control of larvae of *Ae. aegypti* [211]. The major drawback of using neem oil is that its dosage should be considered when applied in the field because neem can cause risks to non-target organisms at higher doses.

Natural pyrethrins are now considered as a potential alternative to synthetic DTT and can overcome hazardous effects of pyrethroids. However, they have major drawbacks which include their high instability and quick degradation in the presence of sunlight. Stability concerns and short duration of their knockdown effect cause inadequate field applications against mosquito populations during the day [212]. However, the application of pyrethrin-based insecticides after sunset against *Culex* and *Anopheles* has shown a decrease in mosquito populations and protection against non-target insects [213]. Pyrethrins are also more effective when used with a synergist. They can be easily degraded before having an impact on mosquitoes, thus should be applied with a synergist of non-synthetic origin. Since pyrethrin-based chemicals are detected via mosquito olfactory organs and processed through olfactory signal transduction mechanisms, pyrethrin-based repellent molecules should be developed and implemented in order to interfere with the host-seeking behavior of mosquitoes for an effective reduction in disease transmission.

Despite our increasing knowledge on plant-based bioactive compounds and their multiple mode of actions on insects, a few of them, such as essential oil-based and neem-based insecticides, have been commercially available for pest management [205]. One of the reasons that causes their limited usage in the field is the formulation problem to overcome phytotoxic effects. The chemical composition of each compound should be formulated in such a way that it should be bioactive to target insects and non-toxic to non-target organisms. In addition, formulation of plant-based bioinsecticides should ensure that it can be produced in large quantities through biomass production of plants and administered in recommended dosages to minimize toxic effects, and biological activity can be maintained for longer shelf-life. As discussed above, microencapsulation and nanoemulsion technologies have benefits in solving formulation issues of phytochemicals. A new formulation in the form of tablets containing a lectin preparation showed mosquitocidal activity against different developmental stages of *Ae. aegypti* mosquitoes, and this formulation method

is proposed as a new control strategy for *Ae. aegypti* populations [214]. Phytochemicals break down rapidly and this causes a need for continuous and more frequent applications in the field for a satisfactory impact on mosquito control. Further studies are needed with the implementation of new methods for the development of effective bioinsecticides from other plant-based bioactive compounds.

9. Conclusions

Mosquitoes are important vectors of devastating diseases, and their hazardous effects are far beyond eradication. The occurrence/reoccurrence of mosquitoes in endemic, non-endemic, and new regions of the world has led to extensive use of synthetic chemicals to control transmission of mosquito-borne diseases. With the increase of resistant mosquitoes and toxicity issues to target and non-target organisms, safer, biodegradable, target-specific alternatives have been considered to replace conventional mosquito control strategies. Phytochemicals have gained importance to overcome mosquito control problems as being considered natural, environmentally safe, less toxic, inexpensive, and, more importantly, less prone to mosquito resistance. Variety of plant extracts have been reported to have mosquitocidal or repellent activity against mosquito vectors, mostly depending on laboratory assays, but there are limitations for their efficacy and applicability in the field. Problems associated with their formulation and commercialization, non-standardization in evaluation of their bioactivities, and their persistence for longer durations should be resolved for development of effective and sustainable methods for their usage. There is no doubt that there are more bioactive compounds that require exploring, and future research should focus on searching for plant-based products with the ultimate goal of deploying them as a reliable remedy to control mosquito populations and mosquito-borne diseases.

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A Three-Pronged Approach to Studying Sublethal Insecticide Doses: Characterising Mosquito Fitness, Mosquito Biting Behaviour, and Human/Environmental Health Risks

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Simple Summary: Extensive research has been carried out to assess the effects of sublethal pyrethroid doses on mosquito fitness and behaviour. Although pyrethroids are mainly used as insecticides, they can also act as repellents, depending on the dosage and/or exposure time. Females and males of two laboratory-reared mosquito species (*Culex pipiens* and *Aedes albopictus*) were exposed to five treatments in the laboratory: three doses of the pyrethroid prallethrin, as well as an untreated and a negative control. Effects on mosquito fitness, mosquito biting behaviour, and human and environmental health were evaluated. Sublethal prallethrin doses were found to decrease mosquito population size, longevity, and biting rate while posing low risks to human and environmental health. Such changes in adult mosquito fitness and behaviour could reduce the ability of mosquitoes to transmit diseases and, consequently, help limit public health risks. Although these promising results suggest sublethal insecticide doses could offer a new approach to controlling species that transmit diseases, more work is needed to identify the proper balance among regulatory requirements, contexts of usage, and human and environmental health benefits.

Abstract: Worldwide, pyrethroids are one of the most widely used insecticide classes. In addition to serving as personal protection products, they are also a key line of defence in integrated vector management programmes. Many studies have assessed the effects of sublethal pyrethroid doses on mosquito fitness and behaviour. However, much remains unknown about the biological, physiological, demographic, and behavioural effects on individual mosquitoes or mosquito populations when exposure occurs via spatial treatments. Here, females and males of two laboratory-reared mosquito species, *Culex pipiens* and *Aedes albopictus*, were exposed to five different treatments: three doses of the pyrethroid prallethrin, as well as an untreated and a negative control. The effects of each treatment on mosquito species, sex, adult mortality, fertility, F1 population size, and biting behaviour were also evaluated. To compare knockdown and mortality among treatments, Mantel–Cox log-rank tests were used. The results showed that sublethal doses reduced mosquito survival, influencing population size in the next generation. They also provided 100% protection to human hosts and presented relatively low risks to human and environmental health. These findings emphasise the need for additional studies that assess the benefits of using sublethal doses as part of mosquito management strategies.

Keywords: prallethrin; insecticide; spatial treatment; mosquito fitness; protection; pyrethroids; *Aedes albopictus*; *Culex pipiens*; life tables

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1. Introduction

Mosquitoes represent a major threat to human health because of their role in the transmission of vector-borne diseases (VBDs). Over the past century, the incidence of mosquito-borne diseases has increased significantly around the world [1–3].

To deal with this threat, researchers are developing novel techniques for use in integrated vector management (IVM) programmes and are focusing on biological, cultural, physical, mechanical, and genetic control methods [4,5]. However, chemical control, such as insecticide use, remains one of the most reliable strategies [6]. Indeed, the use of insecticides in IVM programmes has increased in recent years, reducing human mortality due to VBDs in many countries and thus playing an essential role in efforts to improve public health [7]. Pyrethroids are a key class of insecticides; they are neurotoxins that interfere with nervous system function in arthropods by blocking the closure of sodium channels. As a result, nerve impulses are prolonged, leading to muscle paralysis and, ultimately, death [8]. Worldwide, pyrethroids are the most frequently used insecticide class because they are relatively less toxic to mammals, have a rapid knockdown (KD) effect on the target arthropods, and break down rapidly in the environment due to their high degree of photodegradation [9]. They are widely deployed against agricultural pests, household pests, store-product pests, ectoparasites found on pets and livestock, and vectors of diseases [10].

Biocidal products (BPs) are strictly regulated by governmental authorities. Regulations are based on the physicochemical properties, efficacy, and environmental and human health risks posed by the active substances (ASs) contained in BPs.

Over recent decades, the European Biocidal Product Regulation (BPR) has drastically reduced the number of ASs used in insecticides, primarily as a result of toxicological and environmental concerns and, secondarily, as a result of the high costs associated with justifying the use of existing ASs or registering new ones [11]. In Europe, there are 22 official biocidal product types (PTs). The category PT18 includes the compounds used in insecticides, acaricides, and other arthropod control products that function by means other than repulsion or attraction. The category PT19 includes compounds that control harmful organisms by acting as repellents or attractants, including those that are used to protect human or animal health via spatial treatments and/or application to the skin [12]. Certain compounds, such as pyrethroids, have a dose-dependent effect: depending on the conditions of use, the substance may kill insects (PT18) [13,14] or repel them (PT19). Personal protection products can be found in both categories [13–18]. In Europe, an AS must be registered in both categories to be authorised for both uses. At present, only two ASs have such a dual status: geraniol (CAS number 106-24-1) and *Chrysanthemum cinerariaefolium* extract (CAS number 89997-63-7) [11].

EU efficacy requirements for insecticides used in space treatments stipulate that a formulation/AS dose must kill 90% of exposed insects within 24 h [19], a threshold known as the LD90. Insecticide doses below the LD90 are considered to be ineffective and, therefore, are not authorised. However, there are other issues to consider. First, high levels of mortality require the use of high doses, which conflicts with the constraints imposed by human health risk assessments (HHRAs), whose results are also required for product authorisation.

In turn, a dose is formally defined as sublethal when it induces mortality in less than 50% of exposed insects [20]. While many studies have characterised the effects of lethal pyrethroid doses on different arthropod taxa [21], much remains unknown about how sublethal pyrethroid doses used in space treatments affect mosquito fitness and behaviour or how such doses could be used in IVM programmes [18,22]. However, some studies have revealed that sublethal doses of insecticides could reduce mosquito survival, population sizes [22–24], and biting rates [25,26].

In this study, the effects of prallethrin 94.7% technical grade (CAS number 23031-36-9; PT18), a synthetic Type I pyrethroid, were assessed using two species of laboratory-reared mosquitoes: *Aedes albopictus* and *Culex pipiens*. Both are commonly used in insecticide efficacy tests across the globe. Prallethrin resulted in rapid knockdown (KD) when de-

ployed against household insect pests via indoor space treatments [27]. The work presented here examined the impacts on three variables in particular: (1) mosquito fitness, (2) protection from mosquito bites in humans, and (3) toxicological risks to humans and the environment. In our analyses, we kept in mind the various constraints associated with EU authorisation standards.

2. Materials and Methods

The study was conducted in the Henkel Ibérica Research and Development (R&D) Insect Control Department from February 2020 to March 2021. Three experiments were performed using 5 treatments: 3 sublethal doses of prallethrin (0.40 ± 0.01 mg/h, 0.80 ± 0.01 mg/h, and 1.60 ± 0.01 mg/h), an untreated control, and a negative control.

The lowest dose, 0.4 mg/h, was used as a starting point for defining the 2 other doses. Preliminary research determined that this dose resulted in mortality rates of less than 50% 24 h after exposure (Moreno et al., unpublished data) under experimental conditions similar to those in this study (prallethrin applied via a spatial treatment in the laboratory using 12- to 14-day-old female *Ae. albopictus* and *Cx. pipiens*). Consequently, in this study, the starting dose was doubled (0.8 mg/h) and tripled (1.6 mg/h) to assess the effects of using higher levels of the AS.

To achieve accurate dosing, an electric diffuser composed of polypropylene was used (voltage = 220 V; frequency = 50 Hz; maximum power input = 10 W). It is manufactured by Henkel (model EB03) and is commercially available within the EU. The diffuser consisted of a refillable bottle containing the insecticide and a wick connected to a heater that induced evaporation. The release rate of the diffuser could be modulated by adjusting the heater temperature via the diffuser's 2 settings. There was a normal setting, which released a minimum quantity of insecticide (mg of formula/h), and a maximum setting, which released twice that minimum quantity. Thus, to obtain a dose of 0.4 mg/h, the normal setting was used with 1.1% prallethrin in the bottle. To obtain a dose of 0.8 mg/h, the maximum setting was used with 1.1% prallethrin in the bottle. To obtain a dose of 1.6 mg/h, the maximum setting was used with 2.2% prallethrin in the bottle. Solvent types were the same in all 3 cases. The negative control used a formulation that exclusively contained the solvents. In the untreated control, mosquitoes were not exposed to prallethrin or the solvent formulation.

When the electric diffusers were not being used in the efficacy tests, they were kept running (24 h/day) in an evaporation room (temperature: 25 ± 2 °C) in the department's chemical laboratory.

The quantities (in mg) of the formulations and the prallethrin that evaporated per hour were calculated based on the change in mass over a series of 24-h periods. Evaporation was monitored for a total of 170 h.

The experiments were carried out in a 30-m^3 chamber, as described in Moreno et al. [28,29].

Two mosquito species—*Ae. albopictus* and *Cx. pipiens*—were used. Representatives of *Ae. albopictus* came from a colony at the Entostudio Test Institute (Italy), which Henkel has maintained for the past 8 years. Representatives of *Cx. pipiens* came from an autogenous strain that Henkel has raised at its own facilities for past 14 years; it was originally collected in the field in Barcelona (Spain). Both colonies are known to be susceptible to pyrethroids.

Mosquito-rearing conditions were as follows: a temperature of 25 ± 2 °C, a relative humidity of 60 ± 5%, and a photoperiod of 12:12 (L:D). All the experiments were conducted using 12- to 14-day-old mosquitoes. Although it is standard to estimate mortality in bioassays using mosquitoes of 5–10 days in age, older mosquitoes are more appropriate when changes in biting behaviour need to be evaluated. Thus, mosquito age was standardised for the whole study. Prior to testing, the mosquitoes were separated by species but not by sex. They were allowed to copulate but not to lay eggs. To ensure good activity levels during the experiments, the mosquitoes were given water and a 10% sucrose solution ad libitum before and during the research trials.

2.1. Effects of Sublethal Prallethrin Doses on Mosquito Fitness

The first experiment examined the effects of sublethal prallethrin doses on mosquito fitness and population dynamics. Female and male mosquitoes of both species were subjected to the 5 treatments. In total, 2500 mosquitoes were used: 1250 mosquitoes of each species, of which 625 were females and 625 were males. Each population of 1250 mosquitoes was divided into 10 subgroups of 125 mosquitoes. Five of the subgroups were composed of females and 5 of the subgroups were composed of males. Each subgroup was randomly assigned to 1 of the 5 treatments.

Every day, the chambers were properly cleaned and, before any experiment was begun, the chamber was checked for insecticide contamination. At least 10 mosquitoes were released into the chamber and left there for 30 min. A piece of cotton wool soaked in a 10% sugar solution was provided. Any mortality or KD during this period was noted, and the chamber was considered to be contaminated or in an unsatisfactory state if KD was higher than 10% [30]. A mosquito was considered to be KD if it was lying on its back and was unable to upright itself [31]. If no contamination was detected, the first set of mosquitoes was removed and the experimental set of 125 mosquitoes was released to initiate testing. These latter mosquitoes were given 30 min to acclimate to the chamber and were also provided with a piece of cotton wool soaked in a 10% sugar solution.

After the mosquito acclimatization period, the electric diffuser was run inside the chamber to begin the treatment. The number of mosquitoes that had been KD was counted every 10 min for up to 90 min. At the end of the trial, the mosquitoes were collected using an entomological aspirator and were taken to an insecticide-free room. There, short-term mortality (STM) was assessed at 24 h and 48 h, then long-term mortality (LTM) was assessed once a week until 100% mortality had been reached or 4 weeks had passed, whichever came first. During this period, the mosquitoes were given water and a 10% sucrose solution ad libitum. Additionally, information on locomotor impairment (i.e., loss of legs) was collected. To this end, mosquitoes were observed and classified for 48 h following a given trial. They were placed in the "living" category if they appeared to be morphologically and/or behaviourally unaffected by the treatment (i.e., they were not found lying on their backs and they had all their limbs). They were placed in the "affected" category if they had lost at least 1 leg. They were placed in the "dead" category if they were lying on their backs and failed to react to any external stimuli [32].

In addition to KD, STM, LTM, and locomotor impairment, fertility, egg laying, the ratio of females to males that emerged, and F1 population size were measured. The exact procedures differed slightly between *Cx. pipiens* and *Ae. albopictus*, as described below.

1. *Cx. pipiens* females: Since they came from an autogenous strain, *Cx. pipiens* females did not need to consume blood to lay eggs. Forty-eight hours after the trial, they were given a tray containing water to allow egg laying. During this period, the number of females that drowned was noted for each treatment group.
2. *Ae. albopictus* females: Forty-eight hours after the trial, *Ae. albopictus* females were fed calf's blood using a membrane feeding system (Hemotek, Discovery Workshops, Lancashire, England). Females were given wet paper filters for egg laying, which meant that there was no risk of drowning.

The larval rearing procedure was the same for both species. The eggs were placed in 6-L plastic trays, which were filled with 5 L of water and then labelled by treatment. The larvae developed in the trays under temperature-controlled conditions (25 °C) and were fed rat food (Nanta S.A). Larval density per tray (i.e., 100–120 larvae per litre) was carefully maintained to limit the risk of cannibalism. The water used for larva rearing was not treated with any chemical substances (i.e., anti-algal compounds). The trays were checked every day and additional food was added as needed. Upon reaching the pupal stage, individuals were transferred to the adult emergence containers.

The number of eggs laid over the course of the 4-week post-treatment period was assessed for *Ae. albopictus*, but not for *Cx. pipiens*. In the latter species, eggs are laid in groups (i.e., in egg rafts), making them difficult to count unless separated. For both species,

the number of larvae that reached the third/fourth instar and the percentage of females and males that emerged were determined. The ratio of third/fourth instar larvae to females available for egg laying was also calculated.

2.2. Effect of Sublethal Prallethrin Doses on Mosquito Biting Behaviour

The second experiment examined the effect of sublethal doses on mosquito biting behaviour and, consequently, on host vulnerability. More specifically, it used human volunteers to determine the length of prallethrin exposure that would result in 100% protection.

Six study participants (2 men, 4 women) took part in each trial. They had undergone training to learn how to accurately count mosquito landings. Prior to testing, the skin to be exposed was washed with unscented soap, rinsed with water, rinsed with 70% ethanol or isopropyl alcohol, and then dried with an uncontaminated towel. To ensure that EU guidelines were respected, participants were asked to avoid the use of nicotine, alcohol, fragrances (e.g., perfumes, body lotions, soap), and repellents for 12 h prior to and during testing [19].

Between exposure periods, study participants remained in air-conditioned rooms and kept their activity levels low.

The trials were conducted using only non-blood-fed female *Ae. albopictus*, since the autogenous *Cx. pipiens* strain shows limited interest in feeding on humans.

To ensure good activity levels during the experiment, the mosquitoes were given water and a 10% sucrose solution ad libitum until the trial started.

As in Experiment 1, a preliminary procedure was used to check for insecticide contamination in the chamber. Once the chamber was confirmed to be clean, a pre-treatment trial took place. A total of 20 female mosquitoes were introduced into the chamber [28] and were given 30 min to acclimate. After this period, a study participant entered the chamber with the lower part of their legs exposed; the rest of their body was protected by a light beekeeper's suit. They also wore gloves and white hospital booties [28] (Figure 1). The person remained in the chamber for 3 min [28]. During this time, the number of mosquitoes landing on their exposed skin was recorded. This figure served as a baseline for estimating percent protection following the treatment.

Figure 1. (**a**) The 30-m^3 testing chamber at Henkel's R&D Laboratory. (**b**) Participant wearing a protective suit while inside the chamber.

Percent protection expressed the relative reduction in landings/instances of probing attributable to the treatment for each participant [28]. It was calculated as follows:

$$\% \text{ protection} = (C - T) \times 100/C, \quad (1)$$

where C = number of landings/instances of probing during the pre-treatment trial and T = number of landings/instances of probing during the treatment trial.

Immediately after the pre-treatment trial, the treatment trial began. First, the electric diffuser was switched on inside the empty chamber. After the diffuser had been running for 5 min, the person who took part in the pre-treatment trial again entered the chamber. They remained inside for 3 min, and the number of mosquitoes landing on their exposed skin was recorded. They then left the chamber. This procedure was repeated 10 min and 15 min after trial initiation.

Each participant was exposed once to each of the 3 prallethrin treatments and the 2 controls.

2.3. Assessments of Human and Environmental Health Risks

Toxicological risks were assessed in 2 ways: by estimating human health risks using HHRA models and by estimating environmental health risks.

HHRA models were performed for 2 populations: adults and children 2–3 years old. This work was carried out using ConsExpo Web (v. 1.0.7; [33]), a tool designed by the Dutch National Institute for Public Health and the Environment (RIVM). In ConsExpo Web, certain parameters can be set to a chosen value, while others are fixed.

Because an electric diffuser was used in the experiments, only inhalation exposure was considered. However, it is assumed that some of the AS would end up on the floor, where children 2–3 years old might be crawling, so dermal exposure in children was also considered. It was assumed that there was no oral exposure. Thus, the following ConsExpo models were used: "Inhalation exposure: exposure to spray—spray" and "Dermal exposure: direct contact with product—rubbing off".

Within the inhalation exposure model, the inhalation rate was chosen based on Recommendation 14 of the Biocidal Product Committee (BPC) Ad Hoc Working Group on Human Exposure, which describes the default values to use when assessing human exposure to BPs [34]. In this context, here are the key values that were chosen: first, the exposure duration was 24 h per day (a worst-case scenario). Second, it was assumed that night-time respiration in the bedroom was taking place during all those hours (also a worst-case scenario). The volume of that bedroom, 16 m^3, was one of the values fixed by ConsExpo and was considered to represent yet another worst-case scenario. To determine the exposure duration that would be considered safe for both adults and children, the 3 experimental doses were examined: 0.4, 0.8, and 1.6 mg/h (Table 1).

Within the dermal exposure model, the dislodgeable amount is the quantity of product applied on a surface area that may potentially be wiped off (per unit of surface area) and that thus may be taken up via contact between surfaces and the human skin. A worst-case scenario was assumed: 10% of the applied AS would end up on the floor, and 10% of that amount would be dislodgeable (Table 2).

Table 1. Summary of parameters for the ConsExpo model "Inhalation exposure: exposure to spray—spray".

Parameter	Value
Spray duration	24 h (worst-case scenario)
Exposure duration	To be determined (max. number of hours that exposure remained safe for adults and children)
Weight fraction compound	100% (the prallethrin release rate is considered in the mass generation rate)
Room volume	16 m^3 (fixed value)
Room height	2.5 m (fixed value)
Ventilation rate	1/h (fixed value)
Inhalation rate	16 m^3/d (adult) 10.1 m^3/d (child of 2–3 years old)
Mass generation rate	4.03×10^{-5} g/s (=1.6 mg/h) 2.27×10^{-5} g/s (=0.8 mg/h) 1.02×10^{-5} g/s (=0.4 mg/h)
Airborne fraction	1 (fixed value)
Density, non-volatile	0.85 g/cm^3 (density corrected to formulation)
Inhalation cut-off diameter	15 µm (fixed value)
Aerosol diameter distribution	log normal (fixed value)
Median diameter	8 µm (fixed value)
Coefficient of variation	0.3 (fixed value)
Maximum diameter	50 µm (fixed value)
Body weight	60 kg (adult), 15.6 kg (child 2–3 years old)
Absorption	100% (fixed value)

Chosen and fixed parameter values for the ConsExpo model [33].

Table 2. Summary of parameters for the ConsExpo model "Dermal exposure: direct contact with product—rubbing off".

Parameter	Value
Weight fraction compound	100% (the prallethrin release rate is considered in the mass generation rate)
Transfer coefficient [1]	0.24 m^2/h (fixed value)
Dislodgeable amount	2.93 mg/m^2
Contact time	60 min (fixed value)
Rubbed surface	7 m^2 (fixed value)
Absorption model	Fixed fraction
Absorption	6% (based on experimental results provided by the AS supplier)

AS, active substance. [1] Chosen and fixed parameter values for the ConsExpo model [33].

To assess risks to environmental health, the following assumptions were made: continuous release (24 h/day) of a vapourised liquid containing prallethrin as its AS and the presence of 2 electric diffusers per household, as per the recommendations in the Technical Agreements for Biocides [35].

The European Chemical Agency (ECHA) Emission Scenario Document (ESD) PT18 spreadsheet (regarding indoor diffusers) was filled out in accordance with the instructions contained in the Organisation for Economic Co-operation and Development (OECD) ESD No. 18 [36]. The results were used to estimate potential product presence in wastewater following treatment and cleaning. Exposure values were calculated using the European Union System for the Evaluation of Substances (EUSES) (software v. 2.2.0).

Any additional risks resulting from metabolites were included in the risk assessment.

For each environmental compartment facing exposure, risk was characterised using the ratio of predicted environmental concentrations (PECs) to predicted no-effect concentrations (PNECs). Of greatest concern was the PEC/PNEC ratio for soils.

2.4. Statistical Analysis

To compare the KD and mortality curves based on species, sex, and treatment, Mantel–Cox log-rank tests including pairwise comparisons were carried out in SPSS (v. 15.0.1) for Windows (SPSS Inc., Chicago, IL, USA).

Fisher's exact tests applying the Bonferroni correction method were used to examine treatment effects on mosquito fitness and F1 population size in *Cx. pipiens* and *Ae. albopictus*.

Generalised linear mixed models (GLMMs) were performed to determine how treatment and exposure time affected KD (Poisson error distribution and log-link function; MASS package in R) and percent protection (Gaussian error distribution and identity link function; nlme package in R). The identity of the study participant was included as a random factor. When overall significant differences were detected, pairwise comparisons were performed using *t*-tests with pooled standard deviations and the Bonferroni correction method.

The alpha level was 0.05 for all the statistical analyses.

3. Results

3.1. Effects of Sublethal Prallethrin Doses on Mosquito Fitness

In the first experiment, the following were evaluated: (1) the effects of species, sex, and treatment on KD during the 90-min treatment trial; (2) the percentage of dead and affected mosquitoes 48 h into the post-treatment period; (3) the effects of species, sex, and treatment on long-term mortality (i.e., over the 4-week post-treatment period); and (4) the effects of species, sex, and treatment on fertility, egg laying, and F1 population size.

3.1.1. Effects of Species, Sex, and Treatment on KD during the 90-Min Treatment Trial

All three sublethal doses of prallethrin (0.4, 0.8, and 1.6 mg/h) caused more than 95% of mosquitoes to be knocked out, except in the case of *Cx. pipiens* females (87.2%; Figure 2). The higher the dose, the faster the KD. KD differed between the two control groups and the three prallethrin groups based on species and sex (Figure 2). In the untreated control, there was no KD. In the negative control, only a few male *Ae. albopictus* were knocked down (12.8%; Figure 2b).

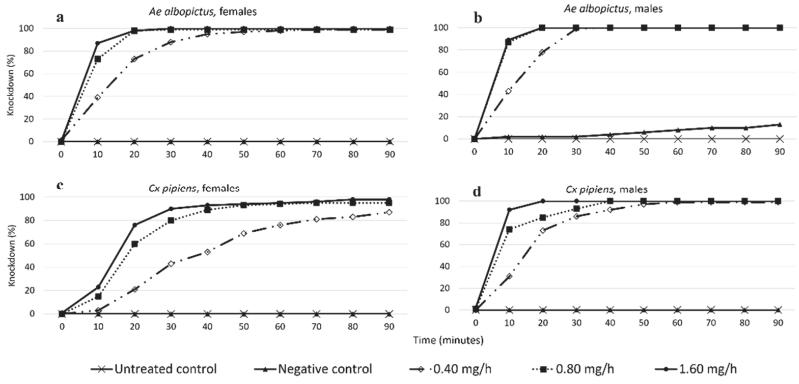

Figure 2. Knockdown over the 90-min treatment trial in Experiment 1 for female and male *Ae. albopictus* and *Cx. pipiens* across the five treatment groups: (**a**) Female *Ae. albopictus*, (**b**) male *Ae. albopictus*, (**c**) female *Cx. pipiens*; and (**d**) male *Cx. pipiens*.

First, KD was compared within species. In *Ae. albopictus*, for both sexes, there was a significant difference in KD between the mosquitoes exposed to the 0.4 mg/h prallethrin dose and the mosquitoes exposed to the 0.8 and 1.6 mg/h prallethrin doses (Table 3). Exclusively in the case of male *Ae. albopictus*, there was no significant difference between the groups exposed to the 0.8 vs. the 1.6 mg/h prallethrin dose. In general, KD was faster

at the higher doses (Figure 3a,b). In *Cx. pipiens*, there were significant differences among all three prallethrin doses for both sexes (Table 3).

Table 3. Comparison of within species knockdown for female and male *Ae. albopictus* and *Cx. pipiens* across the five treatment groups in Experiment 1.

Species	Sex	Treatment Comparisons	χ^2	*p*-Value
Ae. albopictus	Females	Untreated vs. negative control [1]	-	-
		Controls vs. prallethrin groups [2]	-	$p < 0.0001$ in all cases
		0.4 mg/h vs. 0.8 mg/h	34.59	$p < 0.0001$
		0.4 mg/h vs. 1.6 mg/h	63.02	$p < 0.0001$
		0.8 mg/h vs. 1.6 mg/h	6.18	$p < 0.05$
	Males	Untreated vs. negative control	17.03	$p < 0.0001$
		Controls vs. prallethrin groups [2]	-	$p < 0.0001$ in all cases
		0.4 mg/h vs. 0.8 mg/h	61.76	$p < 0.0001$
		0.4 mg/h vs. 1.6 mg/h	65.21	$p < 0.0001$
		0.8 mg/h vs. 1.6 mg/h	0.15	$p = 0.698$
Cx. pipiens	Females	Untreated vs. negative control [1]	-	-
		Controls vs. prallethrin groups [2]	-	$p < 0.0001$ in all cases
		0.4 mg/h vs. 0.8 mg/h	39.88	$p < 0.0001$
		0.4 mg/h vs. 1.6 mg/h	67.29	$p < 0.0001$
		0.8 mg/h vs. 1.6 mg/h	5.49	$p < 0.05$
	Males	Untreated vs. negative control	-	-
		Controls vs. prallethrin groups [2]	-	$p < 0.0001$ in all cases
		0.4 mg/h vs. 0.8 mg/h	25.28	$p < 0.0001$
		0.4 mg/h vs. 1.6 mg/h	102.49	$p < 0.0001$
		0.8 mg/h vs. 1.6 mg/h	22.23	$p < 0.0001$

Pairwise comparisons of knockdown (KD) were carried out using Mantel–Cox log-rank tests in implemented in in SPSS (v. 15.0.1) for Windows (Chicago, SPSS Inc). All the statistical comparisons used an alpha level of 0.05. [1] No statistics were performed because no mosquitoes were knocked down in the controls. [2] Each control group (untreated and negative) was compared with each prallethrin group (0.4, 0.8, and 1.6 mg/h). This row summarises the results. Significant differences were observed between the control groups and the prallethrin groups in all the configurations.

Second, KD was compared between species. At the lowest dose (0.4 mg/h), differences only existed between male *Ae. albopictus* and female *Cx. pipiens* ($\chi^2 = 6.562$, $p < 0.05$). At the intermediate dose (0.8 mg/h), male *Ae. albopictus* experienced significantly faster KD than all the other groups ($p < 0.0001$ for all the comparisons). At the highest dose (1.6 mg/h), there were no differences among female *Ae. albopictus*, male *Ae. albopictus*, and male *Cx. pipiens* (female *Ae. albopictus* vs. male *Ae. albopictus*: $\chi^2 = 0.787$, $p = 0.375$; female *Ae. albopictus* vs. male *Cx. pipiens*: $\chi^2 = 3.645$, $p = 0.056$; male *A. albopictus* vs. male *Cx. pipiens*: $\chi^2 = 1.419$, $p = 0.234$). However, female *Cx. pipiens* experienced significatively slower KD than all the other groups ($p < 0.0001$ for all the comparisons). For example, at 10 min, KD was only 23% for female *Cx. pipiens* but 87–92% for all the other groups (Figure 3).

3.1.2. Percentage of Dead and Affected Mosquitoes 48 h into the Post-Treatment Period

Mosquitoes displayed a variety of fates during the 48 h that followed the trials. Some died, some survived, and yet others remained alive but were clearly affected by the prallethrin. The most obvious sign that surviving mosquitoes had been affected was the partial or complete loss of legs (Figure 3). This effect was observed for all the doses tested, although it was more pronounced at the higher doses (e.g., some individuals lost one or more legs and also died).

Figure 3. Photograph showing a sample of female *Cx. pipiens* that lost legs following prallethrin exposure. The numbers next to the mosquitoes indicate the number of legs lost.

At 24 h into the post-treatment period, dead and affected mosquitoes together accounted for more than 90% of all the mosquitoes in almost all the prallethrin groups. The only exception was female *Cx. pipiens* exposed to the 0.4 mg/h prallethrin dose (41.60% at 24 h and 75.2% at 48 h).

Similarly, at 48 h into the post-treatment period, dead and affected mosquitoes together accounted for more than 90% of all the mosquitoes (females and males combined) in almost all the prallethrin groups. The only exception was *Cx. pipiens* exposed to the 0.4 mg/h prallethrin dose (84.4%).

Dead Adult Mosquitoes. At 24 h into the post-treatment period (Figure 4), male mortality in both species exceeded 90% in almost all the groups exposed to prallethrin. The exception was male *Cx. pipiens* exposed to the 0.4 mg/h prallethrin dose, a group that displayed 80% mortality. In both species, female mortality was lower, especially when mosquitoes were exposed to the 0.4 mg/h prallethrin dose (49.6% and 30.4% for *Ae. albopictus* and *Cx. pipiens*, respectively). At the prallethrin dose of 0.8 mg/h, female mortality was 56% for *Ae. albopictus* and 43.2% for *Cx. pipiens*. At the prallethrin dose of 1.6 mg/h, female mortality was 71.2% for both species.

At 48 h into the post-treatment period (Figure 4), the only increases in male *Cx. pipiens* mortality were seen in the groups exposed to the 0.4 and 0.8 mg/h prallethrin doses (from 80% to 84% and from 95.2% to 96.8%, respectively). Female mortality rates had risen accordingly with higher doses for both species of mosquitoes from 67.7% to 83.2% for *Ae. albopictus* and from 49.6% to 86.4% for *Cx. pipiens* (Figure 4).

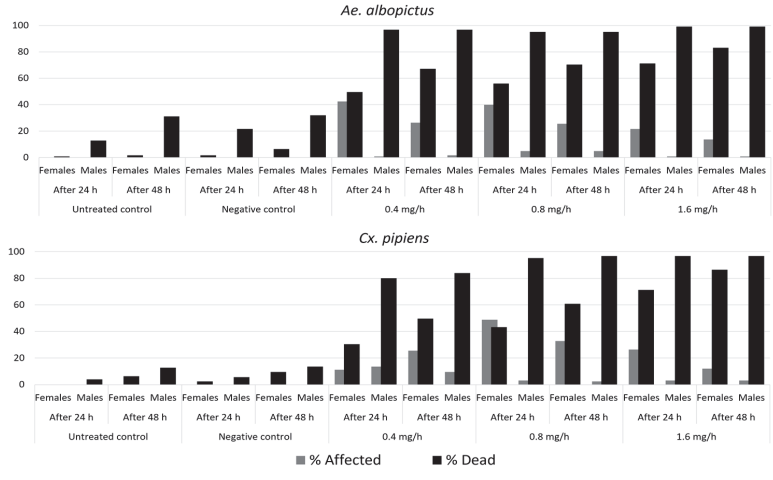

Figure 4. Percentages of affected and dead *Ae. albopictus and Cx. pipiens* at 24 and 48 h into the post-treatment period across the five treatment groups.

Affected Adult Mosquitoes. At 24 h into the post-treatment period, 5% at most (range: 0.8–4.8%) of male *Ae. albopictus* were affected; the rest of the mosquitoes were dead. In the case of female *Ae. albopictus*, there were 42.4% and 40.0% affected mosquitoes in the groups exposed to the 0.4 and 0.8 mg/h prallethrin doses, respectively. At 48 h, these percentages dropped to 26.4% and 25.6%, respectively, largely because the affected mosquitoes had died. For the group exposed to the 1.6 mg/h prallethrin dose, the percentage of affected mosquitoes went from 21.6% at 24 h to 13.6% at 48 h. The same general patterns were seen in *Cx. pipiens*.

At 48 h, the percentages of affected mosquitoes were lower because mortality had occurred. For male *Cx. pipiens*, the group exposed to the 0.4 mg/h prallethrin dose had the highest percentage of affected mosquitos (13.6% at 24 h and 9.6% at 48 h). In contrast, for female *Cx. pipiens*, the percentage of affected mosquitoes increased from 11.2% at 24 h to 25.6% at 48 h for the group exposed to the 0.4 mg/h prallethrin dose; for the groups at prallethrin doses of 0.8 and 1.6 mg/h, these percentages decreased from 48.8% to 32.8% and from 26.4% to 12%, respectively.

Mortality never climbed above 15% in the untreated and negative controls, except in the case of male *Ae. albopictus* (31.2% and 32%, respectively). None of the mosquitoes in the controls showed signs of having been affected (Figure 4).

3.1.3. Effects of Species, Sex, and Treatment on Long-Term Mortality

One week into the post-treatment period, total mortality for female and male *Ae. albopictus* was 90% across all the prallethrin groups; in the controls, however, total mortality was only 28%. For female and male *Cx. pipiens*, the total mortality for mosquitoes exposed to prallethrin doses of 0.4, 0.8, and 1.6 mg/h was 82%, 89.6%, and 94.8%, respectively; for the controls, it was 20.8%.

For both species and sexes, LTM was significantly higher in all the prallethrin groups than in the control groups (Table 4). Within species and sex, LTM did not differ between the untreated and negative controls; it was highest for male *Ae. albopictus* and lowest for female *Ae. albopictus* (Figure 5).

Table 4. Treatment effects on long-term mortality for female and male *Ae. albopictus* and *Cx. pipiens* across the five treatment groups.

Species	Sex	Treatment Comparisons	χ^2	*p*-Value
Ae. albopictus	Females	Untreated vs. negative control	3.15	$p = 0.07$
		Controls vs. prallethrin groups [1]	-	$p < 0.0001$ in all cases
		0.4 mg/h vs. 0.8 mg/h	0.15	$p = 0.69$
		0.4 mg/h vs. 1.6 mg/h	6.40	$p < 0.05$
		0.8 mg/h vs. 1.6 mg/h	5.72	$p < 0.05$
	Males	Untreated vs. negative control	6.32	$p < 0.05$
		Controls vs. prallethrin groups [1]	-	$p < 0.0001$ in all cases
		0.4 mg/h vs. 0.8 mg/h	0.06	$p = 0.80$
		0.4 mg/h vs. 1.6 mg/h	2.07	$p = 0.14$
		0.8 mg/h vs. 1.6 mg/h	3.66	$p = 0.056$
Cx. pipiens	Females	Untreated vs. negative control	3.15	$p = 0.07$
		Controls vs. prallethrin groups [1]	-	$p < 0.0001$ in all cases
		0.4 mg/h vs. 0.8 mg/h	0.15	$p = 0.69$
		0.4 mg/h vs. 1.6 mg/h	6.40	$p < 0.05$
		0.8 mg/h vs. 1.6 mg/h	5.72	$p < 0.05$
	Males	Untreated vs. negative control	1.48	$p = 0.22$
		Controls vs. prallethrin groups [1]	-	$p < 0.0001$ in all cases
		0.4 mg/h vs. 0.8 mg/h	0.93	$p = 0.33$
		0.4 mg/h vs. 1.6 mg/h	4.69	$p < 0.05$
		0.8 mg/h vs. 1.6 mg/h	2.14	$p = 0.14$

[1] Each control group (untreated and negative) was compared with each prallethrin group (0.4, 0.8, and 1.6 mg/h). This row summarises the results. Significant differences were observed between the control groups and the prallethrin groups in all the configurations. Pairwise comparisons of long-term mortality (LTM) were carried out using Mantel–Cox log-rank tests implemented in SPSS (v. 15.0.1) for Windows (SPSS Inc., Chicago, IL, USA). All the statistical comparisons used an alpha level of 0.05.

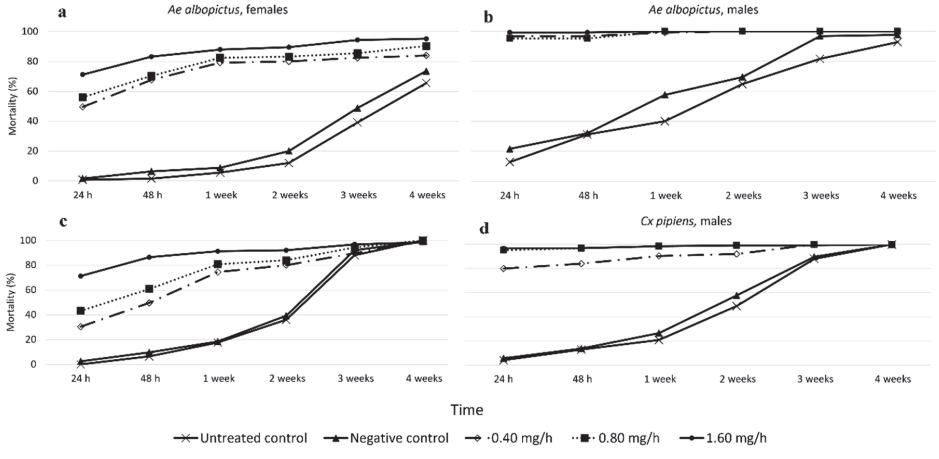

Figure 5. Mosquito mortality during the 4-week post-treatment period across the five treatment groups: (**a**) Female *Ae. albopictus*, (**b**) male *Ae. albopictus*, (**c**) female *Cx. pipiens*, and (**d**) male *Cx. pipiens*. Mortality at 24 h and 48 h is also shown to clarify the relationship between STM and LTM. LTM, long-term mortality; STM, short-term mortality.

LTM did not differ between the groups exposed to the 0.4 and 0.8 mg/h prallethrin doses, regardless of species or sex. It did, however, differ between the groups exposed to the 0.4 and 1.6 mg/h prallethrin doses. It was higher at the latter dose, except in the case of male *Ae. albopictus*—they died equally rapidly across all three doses (100% mortality at 2 weeks post-treatment; Figure 5 and Table 4). In both species, male but not female LTM

was significantly higher in the groups exposed to the 1.6 mg/h prallethrin dose than in the groups exposed to the 0.8 mg/h prallethrin dose (Figure 5 and Table 4).

Sex also affected mortality in the prallethrin groups: LTM was higher for males than females, regardless of species (Figure 5 and Table 4). At 2 weeks post-treatment, male mortality was higher than female mortality by 13–20% for the groups exposed to the 0.4 and 0.8 mg/h prallethrin doses and by 7–10% for the groups exposed to the 1.6 mg/h prallethrin dose.

Species-specific differences in male mortality were present at the lowest prallethrin dose: at 1 week post-treatment, male *Ae. albopictus* exhibited 99.2% mortality, while male *Cx. pipiens* exhibited 90.4% mortality (0.4 mg/h: $p < 0.0001$). There was no such difference for the intermediate prallethrin dose (0.8 mg/h: $\chi^2 = 0.011$, $p = 0.918$) or the highest prallethrin dose (1.6 mg/h: $\chi^2 = 3.806$, $p = 0.051$). Species did not affect female mortality at any of the doses (0.4 mg/h: $\chi^2 = 0.826$, $p = 0.363$; 0.8 mg/h: $\chi^2 = 0.256$, $p = 0.613$; 1.6 mg/h: $\chi^2 = 0.740$, $p = 0.390$).

3.1.4. Effects of Species, Sex, and Treatment on Fertility, Egg Laying, and F1 Population Size over the 4-Week Post-Treatment Period

Culex pipiens. In this part of the experiment, the methodology diverged slightly for the two species because the *Cx. pipiens* strain did not need to consume blood (see the Methods section).

The number of eggs laid by *Cx. pipiens* could not be accurately counted because the eggs formed rafts. Furthermore, some of the rafts were not well assembled. Instead of forming the expected boat-like shape [37], unassembled eggs could be seen on the water surface (Figure 6).

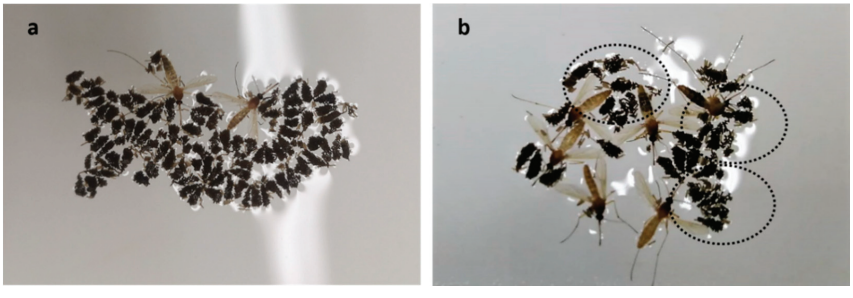

Figure 6. Egg rafts produced by *Cx. pipiens* in the (**a**) untreated control group and (**b**) the group exposed to the 0.8 mg/h prallethrin dose. In (**b**), the poorly assembled egg rafts have been circled to make them easier to identify.

Forty-eight hours after the mosquitoes had been given access to water to lay their eggs, the number of females found dead in the tray was much greater in the prallethrin groups than in the control groups (Fisher's exact tests with Bonferroni correction: $p < 0.001$ for all the comparisons between the control groups (untreated or negative) and each of the prallethrin groups). In the control groups, fewer than 10% of females were found dead, while 23.81%, 38.78%, and 41.18% of females were found dead in the groups exposed to the 0.4, 0.8, and 1.6 mg/h prallethrin doses, respectively (Table 5).

Table 5. Treatment effects on mosquito fitness and F1 population size in *Cx. pipiens*.

Variables Measured		Untreated Control	Negative Control	0.4 mg/h	0.8 mg/h	1.6 mg/h
No. of females alive after 48 h		117	113	63	49	17
% of females found dead in the egg laying tray		8.55	9.73	23.81	38.78	41.18
No. third/fourth instar larvae		4137	3985	2595	2066	637
Ratio of larvae/females		35.36	35.27	41.19	42.16	37.47
% larvae reaching adulthood	Males	36	ND	35.8	39.8	39.7
	Females	32.1	ND	38.8	24.1	30.5
Total no. of adults in F1 population		2816	ND	1936	1320	447
% reduction in F1 population size [1]		-	ND	31.25	53.13	84.13

ND, no data. In the negative control, algae began growing in some of the trays, creating a surface layer that choked off a large percentage of the larvae. This portion of the experiment thus had to be stopped for this group. [1] This metric was calculated for the prallethrin groups based on the total number of adults in the F1 population in the untreated control.

The numbers of larvae to reach the third/fourth instar stage were similar in the untreated control (4137) and in the negative control (3985). Compared with the untreated control, the percentages of reduction in larvae that reached this development stage were 37.27%, 50.06%, and 84.60% for the groups exposed to the 0.4, 0.8, and 1.6 mg/h prallethrin doses, respectively. It is important to note that this result appeared to stem from a smaller number of adults being available to reproduce. When examining the ratio of third/fourth instar larvae to available females, there were no differences among treatments (Table 5).

The percentage of larvae reaching adulthood varied somewhat (64–74% across both sexes), although no treatment effects were observed (Fisher's exact tests with Bonferroni correction: $p > 0.05$ for all the comparisons between treatments). The sex ratio was nearly 1:1 in the untreated control and in the group exposed to the 0.4 mg/h prallethrin dose. The sex ratio was male-biased in the groups exposed to the 0.8 mg/h and 1.6 mg/h prallethrin doses.

There was a pronounced effect of treatment on the F1 population size. Using the untreated control as the standard of comparison, exposure to the 0.4, 0.8, and 1.6 mg/h prallethrin doses reduced the F1 population sizes by 31.25%, 53.13%, and 84.13%, respectively. Declines in population size were significatively different among the three prallethrin groups (Fisher's exact tests with Bonferroni correction: $p < 0.005$ for all the comparisons).

Aedes albopictus. The same data were collected for *Ae. albopictus*, but, in addition, egg number was quantified. As the eggs were laid on wet filter paper, females were not at risk of drowning. In all the groups, including controls, the percentage of females found dead in the egg-laying trays was less than 1%, except for the group exposed to the 0.8 mg/h prallethrin dose (5.41%) (Table 6).

Table 6. Treatment effects on mosquito fitness and F1 population size in *Ae. albopictus*.

Variables Measured		Untreated Control	Negative Control	0.4 mg/h	0.8 mg/h	1.6 mg/h
No. of females alive after 48 h		123	117	41	37	21
% of females found dead in the egg laying tray		0.81	0	0	5.41	0
No. eggs laid		3434	2525	1187	508	356
No. third/fourth instar larvae		1624	1104	639	143	173
Ratio of larvae/females		13.20	9.44	15.59	3.86	8.24
% larvae reaching adulthood	Males	37.32	37.77	33.80	41.26	37.57
	Females	42.86	41.30	46.32	58.04	38.15
Total no. of adults in F1 population		1.302	873	512	110	131
% reduction in F1 population size [1]		-	32.95	60.68	91.55	89.94

[1] This metric was calculated for the prallethrin groups based on the total number of adults in the F1 population in the untreated control.

When examining the ratio of third/fourth instar larvae to available females, no consistent pattern was seen. While there were 15.59 larvae for each female in the group exposed

to the 0.4 mg/h prallethrin dose, this figure was 3.86 and 8.24 in the groups exposed to the 0.8 and 1.6 mg/h prallethrin doses, respectively. A difference was also observed between the controls (untreated control: 13.20 larvae to 1 female; negative control: 9.44 larvae to 1 female; Table 6).

The percentage of larvae reaching adulthood (75–99%) displayed no treatment effects ($p > 0.05$), except the group exposed to the 0.8 mg/h prallethrin dose that differed from the other two prallethrin groups ($p < 0.00001$). The sex ratio was biased towards females, ranged from 0.7 to 1.0, and was unaffected by the treatments.

There was again a pronounced effect of treatment on the F1 population size. Population size declined by 32.95%, 60.6%, 91.55%, and 89.94% in the negative control group and in the groups exposed to the 0.4, 0.8, and 1.6 mg/h prallethrin doses, respectively. Dose significantly affected declines in population size in almost all cases (Fisher's exact tests with Bonferroni correction: $p < 0.00001$ for all the comparisons except that between the groups exposed to the 0.8 versus the 1.6 mg/h dose ($p > 0.05$)) (Table 6).

3.2. Effects of Sublethal Prallethrin Doses on Mosquito Biting Behaviour

Percent protection after 5 min of exposure ranged from 80.07% (±28.38) at the 0.4 mg/h dose to 100% at the 1.6 mg/h dose, but this difference was not significant ($p > 0.05$); (Figure 7. The control treatments provided no protection. At this same time point, KD was null for the two controls; it was 9.33% (±5.39), 17.67% (±49.62), and 51.67% (±7.44) for the 0.4, 0.8, and 1.6 mg/h prallethrin doses, respectively. No significant differences were observed in KD between the groups exposed to the 0.4 versus the 0.8 mg/h dose ($p > 0.05$); there were significant differences in KD at 5 min for the groups exposed to the 0.4 versus the 1.6 mg/h dose and the 0.8 versus the 1.6 mg/h dose ($p < 0.00001$ in both cases). After the diffuser had been running for 15 min, 100% protection was seen in all the prallethrin groups ($p > 0.05$). KD remained null for the two controls; it was 80.17% (±10.25), 95.83% (±4.92), and 100.00% (±0.00) for the 0.4, 0.8, and 1.6 mg/h prallethrin doses, respectively (Figure 7). There was a significant difference between the groups exposed to the 0.4 versus the 1.6 mg/h dose ($p < 0.05$) but not between the groups exposed to the 0.4 versus the 0.8 mg/h dose ($p > 0.05$) or the groups exposed to the 0.8 versus the 1.6 mg/h dose ($p > 0.05$).

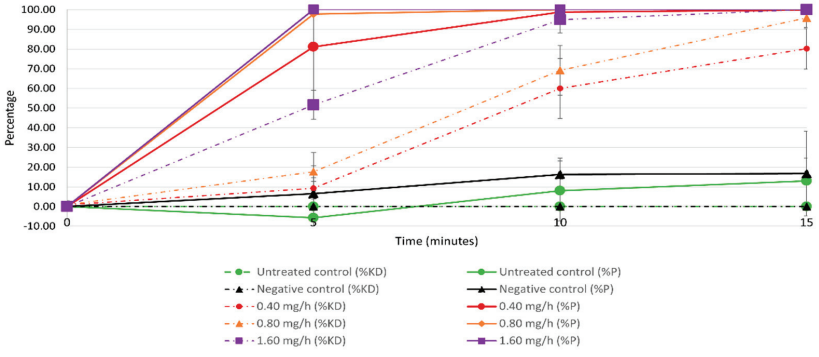

Figure 7. Percent protection (%*p*) and knockdown (%KD) over time for *Ae. albopictus* across the five treatment groups in Experiment 2.

When assessing percent protection, there were no differences between the untreated and negative controls at any of the time points (i.e., $p > 0.05$ at all time points). The same pattern was seen for KD ($p > 0.05$ at all time points).

When the relationship between KD and percent protection was examined, it was found that once KD reached 10%, protection never dropped below 90%. In the controls, negative percent protection values were observed because there were greater numbers

of landings during the treatment trial than during the pre-treatment trial. KD was not observed in the control groups (Figure 8).

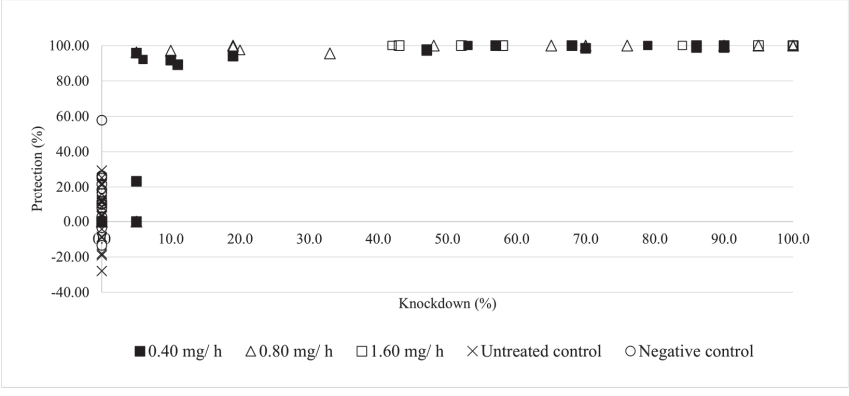

Figure 8. Relationship between knockdown and percent protection for *Ae. albopictus* across the five treatment groups in Experiment 2.

3.3. Assessments of Human and Environmental Health Risks

The HHRA models found that if a prallethrin dose of 1.6 mg/h were to be used, adults could be exposed for 24 h per day, but children could only safely be exposed for 12 h per day. At a prallethrin dose of 0.8 mg/h, children could be exposed for a maximum of 20 h per day. At the lowest dose, 0.4 mg/h, both adults and children could be exposed for 24 h per day.

In the environmental risk assessment, PECs and PNECs were determined for different environmental compartments. When the PEC/PNEC ratio is greater than 1, the AS poses a risk. If prallethrin were to be used 24 h per day and released using two diffusers per household, it would not be safe to use a dose of 1.6 mg/h (PEC/PNEC ratio for soils: 1.34). However, lower doses—0.8 and 0.4 mg/h—would be safe under the same usage conditions (PEC/PNEC ratio for soils: 0.75 and 0.33, respectively).

4. Discussion

When used at sublethal doses applied via a diffuser-mediated spatial treatment, the pyrethroid prallethrin affected the fitness of laboratory-reared *Cx. pipiens* and *Ae. albopictus* adult mosquitoes. The insecticide influenced short- and long-term mosquito mortality, physical status, and egg laying. As a result of reduced mosquito fitness, the size of the F1 population declined in the three prallethrin groups in both species. The mosquitoes' behaviour was also altered. Biting was completely inhibited in as little as 15 min, offering 100% protection to potential human hosts. The modelling revealed that lower doses pose less risk to human and environmental health.

More than 50% of female mosquitoes were still alive 24 h after exposure to the 0.4 and 0.8 mg/h prallethrin doses; this figure was 28.8% for the 1.6 mg/h prallethrin dose. Although technically alive, these mosquitoes nonetheless suffered severe damage to their locomotor systems (e.g., they were missing up to five legs; Figure 4). Previous studies have also observed this phenomenon in response to insecticide exposure [38,39]. Leg loss could theoretically have a major impact because mosquitoes use their legs for a wide variety of functions, including locomotion, mechanical support (e.g., remaining on the water surface, laying eggs), chemical communication, sensory perception of the environment, and protection from desiccation [40,41]. However, other work found that insecticide-induced leg loss did not significantly affect the success of blood feeding or egg laying [38]—mosquitoes with fewer legs were still able to bite humans and reproduce,

maintaining their life cycle. The mortality of adult mosquitoes increased in the days following prallethrin exposure, a pattern that may have been due, entirely or in part, to the insecticide's irreversible effects on the nervous system. For example, the mosquitoes may have been unable to metabolise the AS [42], or they may have struggled to seek out and/or acquire food [43]. Furthermore, female *Cx. pipiens* were found dead in the water when eggs were counted at 48 h post-treatment. It may be that, having lost legs, they were unable to remain on the water surface when laying eggs [38,44]. The combined percentage of dead and affected mosquitoes exceeded 90% for almost all groups at 24 h into the post-treatment period. The only exception was the female *Cx. pipiens* exposed to the 0.4 mg/h prallethrin dose (24 h: 41.6% and 48 h: 75.20%). According to European efficacy guidelines, for an AS/BP to be officially classified as an insecticide useable in spatial treatments, it must kill 90% of females within 24 h of exposure [30]. None of the doses tested in this study would meet the minimum requirements allowing insecticide authorisation; repellent use would also be prohibited because the compound is not authorised for that purpose. It should be noted that the 24-h window of observation means that authorisation decisions are based solely on "immediate" mosquito mortality. Therefore, the long-term mortality observed in this study would not be taken into account for authorisation purposes, even if the mosquitoes were to be "moribund/affected" at 24 h and then finally die at 48 h [30]. OECD guidelines provide specific instructions for such situations: "*Insects in [a] supine position and those [in a] ventral position without [the] ability to move forward and exhibiting uncoordinated or sluggish movements of legs are classified as moribund. Moribund test organisms are counted as dead, if they die within the test duration*" [32].

Looking at the long-term mortality, starting at 1 week into the post-treatment period, total mortality (females and males) for both species for all the prallethrin doses was 80–95%. The lowest level of LTM, 82.4%, was seen in the *Cx. pipiens* exposed to the 0.4 mg/h prallethrin dose. The highest level of LTM, 94.8%, also occurred in *Cx. pipiens*, in the mosquitoes exposed to the 1.6 mg/h prallethrin dose. In contrast, in the controls, total LTM was lower than 30% for both species. At the end of the first experiment (i.e., 4 weeks into the post-treatment period), even doubling the dose from 0.4 to 0.8 mg/h did not significantly increase LTM, regardless of species or sex. However, LTM did climb when tripling the dose from 0.4 to 1.6 mg/h. It should be noted that the mosquitoes in all the prallethrin groups had significatively higher LTM than the mosquitoes in all the control groups (Figure 1); there was no difference in LTM between the untreated and negative controls. Additionally, the first experiment showed that females were less susceptible than males to prallethrin (Figure 5). Sex-specific differences in susceptibility to insecticides have been seen before in laboratory populations [45] and field populations [46]. In both cases, males were found to be more susceptible than females. It is hypothesised that this difference is related to the males' smaller size and/or greater physiological susceptibility [47,48]. Nevertheless, it should be noted that, in all treatments, females survived significantly longer than did males. Consequently, biological factors appear to also influence mosquito mortality and survival.

Prallethrin exposure caused a marked decline in the size of the F1 population. The higher the dose, the larger the decline, which reached a maximum of 80–90% for both species. The above pattern likely stemmed from the higher mortality in exposed mosquitoes. The insecticide did not appear to affect female fertility in *Ae. albopictus*, given that, across treatment groups, there was consistency in the ratio of larvae to females (see Table 6). Additionally, because eggs could be accurately counted in this species, it was possible to confirm that the percentage of eggs that developed into third/fourth instar larvae was also fairly consistent (43.36% in the negative control and 53.8% for mosquitoes exposed to the 0.4 mg/h prallethrin dose), although it was rather low for the group exposed to the 0.8 mg/h prallethrin dose. For *Cx. pipiens*, it was hypothesised that insecticide exposure could affect egg viability via its impacts on raft assemblage (Figure 7) [37]. This hypothesis was based on the results of previous research. For example, Bibbs et al. [22] discovered that sublethal doses of the pyrethroid transfluthrin could cause chorion collapse in *Ae. aegypti* eggs, rendering them non-viable. In this study, the eggs of *Ae. albopictus* did not show any

external signs of damage that could suggest issues with their viability. However, no clear conclusions could be drawn from the ratio of larvae to females, which ranged between 35.27 for the untreated control and 42.16 for the mosquitoes exposed to the 0.8 mg/h prallethrin dose.

Other studies have shown that exposure to pyrethroid vapours (i.e., those of metofluthrin or transfluthrin) at sublethal doses can affect female fertility and egg laying by causing declines in egg viability [22,24] and larval survivorship [24]. However, in those studies, the mosquitoes were placed in small containers (<500 cm^3), not in a large chamber as in this study (30 m^3). Room size and/or the distance of the mosquitoes from the source of the insecticide could influence treatment efficacy. Another factor that could have an influence on the results is whether the mosquitoes were free flying or in cages. For example, any equipment used to constrain the mosquitoes could restrict the aerial diffusion of the AS [15,23,49]. Here, mosquitos could fly freely within a large chamber. As a result, it was impossible to control mosquito distance from the diffuser, but such a design probably better replicates AS use in real life and their influence on mosquitoes. Thus, returning to this study's results, the testing conditions used did not allow clear conclusions to be made about the effect of sublethal prallethrin doses on mosquito fertility. Further research is needed to determine whether more prolonged prallethrin exposure (i.e., longer than 90 min) could yield more definitive results.

With regards to biting behaviour, even the lowest dose of prallethrin, 0.4 mg/h, reduced the host-seeking efficiency of mosquitoes, resulting in 100% protection and 80–100% KD after 15 min. However, it was not necessary to reach 80% KD to greatly inhibit biting (Figure 8). In fact, even when just 10% of the population was knocked down, the level of protection against mosquito bites was approximately 90% (Figure 8). This result can be explained by prallethrin's effects. At low doses/exposure times, the insecticide causes mosquitoes to become disoriented. At higher doses/exposure times, the effects on the nervous system are more pronounced. Certain mosquitoes are knocked down, while others experience a dramatic impairment of their host-seeking abilities [50,51]. Although the importance of modifying vector behaviour has been recognised for decades, the utility of this tool remains greatly underestimated from the standpoints of both BP authorisation and disease control efforts.

When assessing an AS, it is also crucial to consider any risks to human and environmental health. The toxicological results showed that only the lowest dose (0.4 mg/h) would allow 24-h insecticide use by adults and children indoors while also limiting the environmental risks. However, such a low dose would not be authorised in this context of use under current EU requirements for insecticides, which only focus on immediate mortality and do not consider additional data such as LTM and/or beneficial behavioural modifications. Further studies are needed to define how much longer exposure would need to last at low doses for the compound to meet European efficacy requirements (i.e., 90% mortality within 24 h).

Worldwide, pyrethroids are commonly used to control insects, both at the individual level and the environmental level; for example, they are frequently part of IVM programmes [52]. Extensive research has been carried out to assess the effects of sublethal pyrethroid doses on mosquito fitness [22,24,49] and behaviour [23,53,54]. Although pyrethroids are used as insecticides, they can also function as repellents when certain doses or exposure times are used. If insecticides have appropriate levels of volatility, they can be used in space treatments at sublethal doses. Examples of such insecticides include metofluthrin [24,49], transfluthrin [22,55], d-allethrin [25], or prallethrin, the compound studied here [54]. Less volatile insecticides such as permethrin or deltamethrin function better as contact repellents [26,56,57]. For the latter group to be effective, mosquitoes must come into direct contact with the AS, which is possible when insecticides are applied to netting, for example [58,59]. In the case of space treatments, mosquitoes can detect the airborne compounds and avoid entering the treated area [18,60,61]. Multiple studies have demonstrated the efficacy of these insecticides at low doses and their potential benefits for

public health and mosquito control efforts [22–25,49,60]. However, in Europe, they are only authorised for use as insecticides, which greatly limits their potential utility [11].

This study found that sublethal prallethrin doses applied indoors via a spatial treatment had a significant effect on mosquito mortality and biting behaviour. This approach could thus potentially be used to reduce the vector capacity of mosquitoes and, consequently, public health risks. Although the research results presented here are promising, more studies on this complex topic are obviously needed. First, this study utilised two mosquito strains that have been bred exclusively in the laboratory for several years. As a result, it is unknown how well the above findings may reflect the reality in wild mosquito populations. Further studies addressing this issue should be performed. There are other directions that future research can take to explore the benefits and/or limitations of using sublethal doses of pyrethroids in mosquito control efforts. A logical tack to take is to further examine the usefulness of sublethal pyrethroid doses in IVM programmes by evaluating how compounds used as spatial treatments operate under field conditions. Although the concentration of the AS in the air is much lower, the environmental risks could be greater. When considering outdoor applications, an important factor to examine is the development of resistance in mosquito populations via continuous exposure to sublethal pyrethroid doses. Potential shifts in vector sensitivity or susceptibility under such conditions must be explored to assess the likelihood of this potential side effect [62–64].

It is essential to remember that, in the future, a major constraint will be the costs associated with justifying the use of, evaluating the efficacy of, and registering new compounds or compound uses under the BPR [65]. By utilising new evaluation parameters and/or adopting new authorisation paradigms (i.e., LTM and mosquito biting behaviour), it should be possible to exploit currently authorised compounds in new ways [66]. As a result, it may be possible to eliminate the above barrier to innovation and thus help ensure the continued availability of compounds that can effectively control mosquitoes while limiting risks to human and environmental health.

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Institutional Review Board Statement: The work conducted herein was approved by the ethics committee of Henkel AG & Co. KGaA. It meets the company's corporate standards, which ensure health, safety, and respect for the environment, as well as the protection and ethical treatment of all study participants. The study was also conducted in accordance with the ethical principles of the Declaration of Helsinki. Participants were recruited and signed a written informed consent form that explained the study's purpose and procedures as well as the participants' roles and responsibilities; the form also notified participants of their right to withdraw or refuse to take part in the study at any point.

Data Availability Statement: The datasets generated during and/or analysed during the study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: The authors declare no conflict of interest.

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Review

Implications of Sublethal Insecticide Exposure and the Development of Resistance on Mosquito Physiology, Behavior, and Pathogen Transmission

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Simple Summary: Mosquitoes are one of the greatest threats to human lives; they transmit a wide range of pathogens, including viruses that cause lethal diseases. Mosquitoes are found in both aquatic (as larvae or pupae) and terrestrial (as adults) environments during their complex life cycle. For decades, insecticides have been systematically used on mosquitoes with the aim to reduce their population. Little is known about how the stress resulting from the exposure of mosquitoes to insecticides impacts the tri-partite relationship between the mosquitoes, their vertebrate hosts, and the pathogens they transmit. In this work, we review existing experimental evidence to obtain a broad picture on the potential effects of the (sub)lethal exposure of hematophagous mosquitoes to different insecticides. We have focused on studies that have advanced our understanding of their physiological and behavioral responses (including the mechanisms behind insecticide resistance) and the spread of pathogens by these vectors—understudied but critically important issues for epidemiology. Studying these exposure-related effects is of paramount importance for predicting how they respond to insecticide exposure and whether this exposure makes them more or less likely to transmit pathogens.

Abstract: For many decades, insecticides have been used to control mosquito populations in their larval and adult stages. Although changes in the population genetics, physiology, and behavior of mosquitoes exposed to lethal and sublethal doses of insecticides are expected, the relationships between these changes and their abilities to transmit pathogens remain unclear. Thus, we conducted a comprehensive review on the sublethal effects of insecticides and their contributions to insecticide resistance in mosquitoes, with the main focus on pyrethroids. We discuss the direct and acute effects of sublethal concentrations on individuals and populations, the changes in population genetics caused by the selection for resistance after insecticide exposure, and the major mechanisms underlying such resistance. Sublethal exposures negatively impact the individual's performance by affecting their physiology and behavior and leaving them at a disadvantage when compared to unexposed organisms. How these sublethal effects could change mosquito population sizes and diversity so that pathogen transmission risks can be affected is less clear. Furthermore, despite the beneficial and acute aspects of lethality, exposure to higher insecticide concentrations clearly impacts the population genetics by selecting resistant individuals, which may bring further and complex interactions for mosquitoes, vertebrate hosts, and pathogens. Finally, we raise several hypotheses concerning how the here revised impacts of insecticides on mosquitoes could interplay with vector-mediated pathogens' transmission.

Keywords: host-seeking behavior; insecticide exposure; insecticide resistance; mosquito; pathogen transmission

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1. Background

Vector-borne diseases can cause severe harm to human health, including morbidity and mortality depending on the pathogen infection, diagnosis, and treatment quality available for infected individuals [1,2]. For years, the scientific community has worked to develop ways to mitigate the effects of these diseases, and one of the main approaches used is the reduction of vector populations [2,3]. The principal vectors of many human pathogens are mosquitoes. For example, the mosquito *Aedes aegypti* (Diptera: Culicidae) is capable of transmitting multiple pathogens, including the Dengue, Zika, and Chikungunya viruses [4,5]. Furthermore, several mosquito species of the genus *Anopheles* are responsible for transmitting the protozoan *Plasmodium*, which causes malaria, as well as the worms that cause lymphatic filariasis. These worms are also transmitted by other mosquito species, including *Culex quinquefasciatus* (Diptera: Culicidae). The abilities of these pathogens to be transmitted by widely distributed vectors explain their worldwide distributions. For instance, the Dengue virus is now present in at least 129 countries and was estimated to infect around 390 million people every year [1]. Furthermore, the *Plasmodium*-caused disease malaria was considered endemic in at least 87 countries, with 229 million cases reported for 2019 in the latest 2021 report [2].

Several approaches have been used to reduce vector populations, which spans from the reductions of breeding sites up to the use of insecticides to control the abundances of both the vector's immature and adult stages [6–8]. Pyrethroids, synthetic analogs derived from pyrethrins (naturally occurring compounds present in the flower buds of certain *Tanacetum* species), are the most widely used group of insecticides, [9,10]. Pyrethroids and pyrethrins act by disrupting the functioning of voltage-gated sodium channels in insects. Pyrethroids are stabler and more toxic to insects than pyrethrins are and cost less to produce [8,11,12]. Organophosphates and carbamates are two other nerve-active insecticide groups that target acetylcholinesterase enzymes, and consequently are as quick-acting as pyrethroids [13]. Finally, two other relatively slow-acting groups of insecticides commonly used against mosquitoes are insect growth regulators (e.g., pyriproxyfen, which attacks the hormonal balance to disrupt growth and development) and biorational insecticides (e.g., *Bacillus thuringiensis* var. *israelensis* (*Bti*), which targets the midgut) [14,15].

To control adult mosquitoes, several insecticide application techiniques can be used, such as indoor residual sprays (IRS), long-lasting insecticidal nets (LLIN), aerosol sprays, and fumigations [7,8,16]. The IRS and LLIN are the most commonly used methods for the control of *Anopheles* spp., and were responsible for significant decreases in the number of malaria cases in Africa from 2000 to 2019 [2,6,8,17]. However, successive reports of insecticide resistance, especially to pyrethroids, have caused uncertainty regarding the current progress in vector control and put the sustainability of the continuous use of IRS and LLIN in doubt [6,8,16]. Despite the promising launch of a large-scale pilot vaccination program with a first vaccine candidate for malaria in 2019 [17], the report of an increase of 12 million malaria cases per year from 2014–2019, is an indication of delayed progress in malaria control [2]. Pyrethroids are also used to control *Ae. aegypti* and other culicid mosquitoes, and this has unsurprisingly imposed strong pressure for the selection of resistant populations [18–22]. Resistance has also been reported against other insecticide groups, including organophosphates, insect growth regulators, and *Bti* [23,24]. However, since research on the effects of these compounds has been scarce, they have been discussed to a lesser extent than pyrethroids in this review. Interestingly, despite growing reports of pyrethroid resistance globally and the intensive use of insecticides against mosquitoes, the ways in which sublethal exposure to these compounds and resistance-associated population genetic changes affect the transmission of pathogens have remained elusive [25,26].

In the current review, the multiple facets of insecticide exposure effects on mosquitoes (Culicidae) that can interplay with their vector competence (i.e., the ability of a vector to transmit a pathogen [27], correlated with overall pathogen transmission risks) were highlighted (Figure 1). The main text is divided into Sections 2 and 3. Section 2 contains information on the direct and acute effects of sublethal concentrations on individuals and

populations; it is further divided into two parts, each dedicated to effects of exposure on adult and larval mosquitoes. Section 3 reviews the effects of changes in population genetics caused by selection for resistance after exposure to high doses of insecticides. Section 3 is organized into three subsections, each discussing one of the three major resistance mechanisms: target site mutations, metabolic resistance, and behavioral resistance. Understanding both the sublethal and lethal effects of insecticide exposure on the biological and behavioral responses of mosquitoes, especially those impacting their blood meal-related activities—a key point in pathogen transmission—can lead to the development of novel approaches that provide comprehensive conclusions linking control strategies to epidemic risks.

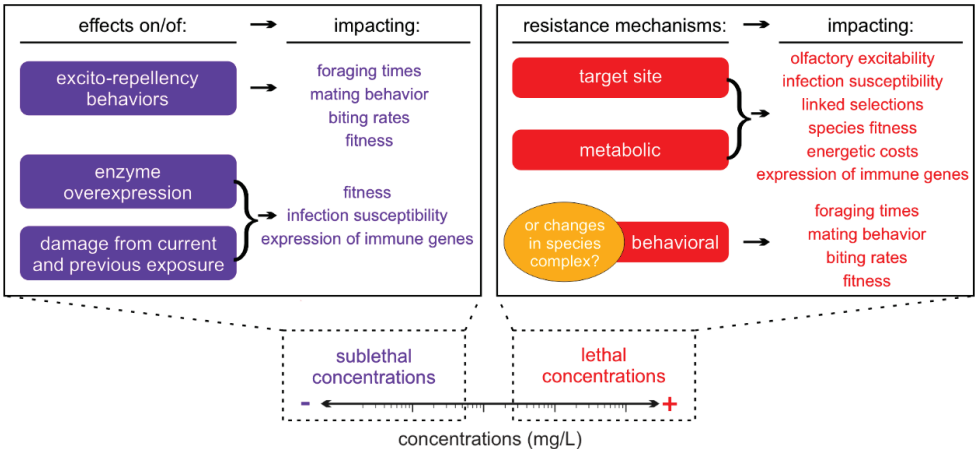

Figure 1. Summary of potential impacts of different insecticide concentrations on mosquito physiology and behavior.

2. Changes in Pathogen Dissemination by Mosquitoes That Survived an Insecticide Exposure

Insecticides, like any other xenobiotics, can directly impact both the biology and behavior of mosquitoes, after non-lethal exposures. Upon contact with the insects, insecticide molecules can induce behaviors, or cause energy-consuming biochemical reactions before causing lethality [28]. These behaviors or energetic costs can reduce insect fitness by disturbing feeding or reproductive behaviors, and thus interfere with the vector competence [29,30]. For example, only sufficiently "aged" female mosquitoes can transmit the malaria-causing protozoan *Plasmodium falciparum*, which takes about 10 to 15 days to complete its life cycle within the vector and be ready for transmission to humans [26]; therefore, decreases in vector survival time resulting from sublethal insecticide exposure or lower blood meal volumes would reduce pathogen transmission [29,31].

Herein, we structured separate sections for different responses of the immature and adult life stages. In the case of adults, two different situations resulting in sublethal exposures have been considered: (a) reduced exposure on treated surfaces because of irritability (i.e., stimulus-dependent repellency) [32–34]; and (b) exposure to low insecticide concentrations due to the expected reduction of insecticide residuals in IRS and LLIN [35]. Regarding the sublethal exposure of larvae, sublethal exposures are results from the direct application of insecticides that dilute in water bodies [36,37] or through indirect contamination of aquatic systems by insecticides in runoff [38]. Nevertheless, for both cases (i.e., larvae and adults), most of the impacts of exposures to sublethal concentrations occurred through the toxicity of the insecticide itself and its cascading effects on subsequent generations [39,40]. These sublethal responses contrast with the impacts of lethal exposures,

which selected for resistant populations (discussed in Section 3) and thus had presented long-term and stable population genetic effects, even if insecticide use is suspended [32,41].

2.1. Sublethal Exposure of Adults to Insecticides

As mentioned above, pyrethroids in IRS and LLIN have been the most commonly used methods for controlling adult mosquitoes. Pyrethroids target voltage-gated sodium channels in insects. They act by prolonging their opening state and causing repetitive firing (type I pyrethroids) or long membrane depolarization (type II pyrethroids); both lead to convulsions, subsequent paralysis, and eventually death [11,12]. Structurally, type II pyrethroids harbor an α-cyano moiety at the phenyl benzyl alcohol position that is absent in type I pyrethroids. The presence of this α-cyano moiety confers type II pyrethroids higher toxicity than type I pyrethroids [9].

In addition to the pyrethroid lethal effects, several mosquito species (e.g., *Anopheles* spp. and *Ae. aegypti*) are irritated by contact with pyrethroid molecules [34,42–44]. Studies showed that certain populations of *Anopheles* species also respond to the presence of pyrethroids (especially type I, which are often more volatile than type II) before making physical contact with a treated surface, suggesting their ability to recognize volatile particles by olfaction [33,45]. These excito-repellent behaviors allow sublethally exposed mosquitoes to escape from insecticide residues [43]. This escape behavior could lead to the reduction of human bites inside homes, which would reduce pathogen transmission rates. However, the outcome of this irritability on mosquito physiology and the potential risk of mosquitoes being present in greater numbers outside of homes, where people cannot be protected by bed nets or IRS, should also be considered as potential factors increasing the transmission risks [44].

Mosquitoes' foraging and learning abilities depend on the normal functioning of their neural system, which can be modulated by both their physiological status and environmental cues [46–50]. Therefore, the direct impacts of sublethal exposures to pyrethroids on most of the insect's abilities is expected to be dependent on their sensory and neural systems, especially considering that not only pyrethroids but also several other insecticides target the mosquito nervous system [8]. Cohnstaedt and Allan [51] demonstrated that female mosquitoes of *Ae. aegypti*, *Cx. quinquefasciatus*, and *Anopheles albimanus* needed more time to initiate a flight response to host cues, flew slower, and had higher flight turning rates after being exposed to two pyrethroids (i.e., deltamethrin and permethrin, type II and I, respectively). This impaired flight ability is expected to interfere with insect mobility and reproduction, but it is not known to what extent this impairment may affect the transmission of pathogens.

When considering the reproductive and olfactory abilities of insects, the impacts of sublethal concentrations of insecticides were usually reported to be negative for their fitness [52–55]. However, studies had also reported the absence of these negative impacts, and in less common circumstances, the occurrence of beneficial effects for the insect's fitness [56–60], the latter which is termed 'hormetic effect', and are usually but not exclusively related to pyrethroids [61,62]. Interestingly, the majority of the records of sublethal effects in mosquitoes have demonstrated only negative results after insecticide exposures, with no hormesis identified [35,63]. The fact that there are few studies reporting impacts of pyrethroid sublethal exposure in mosquitoes, and that even in these studies the doses used are high enough to cause mortality of at least part of the exposed individuals (e.g., LD_{10} or higher [63]) might explain the lack of evidence for pyrethroid mediated hormetic effects, which is usually reached at much lower and narrow concentration ranges.

Several species have been found to constitutively overexpress detoxifying enzymes as a tolerance or resistance mechanism [64–67]. However, the overexpression of such enzymes can also occur only after contact with sublethal concentrations of insecticides in susceptible individuals. This phenomenon of increased expression of detoxifying enzymes was observed in a susceptible population of *Anopheles coluzzii* after sublethal exposure to pyrethroids [68], and this is probably a common response among insects [69–71]. This

overexpression of enzymes leads to energetic costs that may negatively impact several life-history traits related to fitness. However, the fitness costs of induced metabolic responses in mosquitoes have only been assessed for constitutive metabolic resistance, as mentioned in the next section (i.e., Section 3.2) [72,73]. Additionally, an impaired mosquito would probably have its flight abilities reduced, as it had been shown for other insect species [51,74–76], and have most of their biological and physiological parameters impacted.

Pathogens, such as the Dengue and Zika viruses, need to overcome the midgut barrier to disseminate throughout the insect hemolymph and eventually reach the salivary gland before they can be transmitted. Sublethal exposure to a pyrethroid insecticide (i.e., bifenthrin) increased the amount of Zika virus that passed through the midgut of *Aedes albopictus* females [77]. The higher dissemination ability in exposed females may be related to differential energy resource allocation caused by insecticide exposure that reduces the insect imme response, facilitating the dissemination of the virus. Another study showed that this increased dissemination of pathogen happens in the early days after the ingestion of infected blood, but in later days after an infected blood meal, the viral dissemination in the control infected mosquitoes also increases, and both mosquito groups end up presenting 100% viral dissemination [78]. Further studies are needed to confirm the underlying mechanisms that drive the observed differential pathogen dissemination rate found by these studies and to assess how would insecticide exposure impacts pathogen dissemination within mosquito and transmission risks.

Finally, *Bti*, which is normally used in larval control, appears to influence adult *Ae. albopictus* to detect cues in the water body to which *Bti* is added, since its presence induced an increase in the oviposition behavior of this mosquito [79]. However, in the same study, it was demonstrated that the toxicity of *Bti* to emerging larvae could be maintained for several days without any change in efficacy [79].

2.2. Sublethal Exposure of Larvae to Insecticides

Many studies that investigated the effects of sublethal concentrations of pesticides on larval mosquitoes have reported that the physiology of an individual is not reset during metamorphosis to enable it to negate these effects in adults [80,81]. The effects of physiological stressors during the immature developmental stage on the adult can also be difficult to interpret, as they may have multiple outcomes, such as longer development times, male-biased sex ratios, and higher emergence rates and body sizes [82–85] or they offer null impacts, depending on the xenobiotics [86,87].

Sublethal exposures to both pyrethroids or organophosphates in the fourth larval instar caused reductions in adult longevity, fecundity, and wing length in *Cx. quinquefasciatus* [82]. Furthermore, in this case, the fewer eggs laid by treated females were also smaller than the eggs of control females [82], which would impact the next generation's fitness as well. These impacts may be mediated by changes in larval swimming behavior induced by sublethal exposure to these pesticides, as was demonstrated in *Ae. aegypti* [37]. In addition to energy loss due to faster wriggling movements, the impact on the feeding ability of these insects would also explain the longer developmental period necessary to reach the pupal stage [37]. On the other hand, sublethal exposure to malathion in first-instar larvae of *Ae. aegypti* resulted in larger adult females at 20 ºC, but not at 30 ºC, demonstrating that other environmental factors during exposure can also shape the nature of the effects of sublethal insecticide exposures [88]. In this case, the reduced competition resulting from the elimination of small or more susceptible larvae was proposed as an explanation for the larger-sized females that developed from larvae grown at the lower temperature [88]. Nonetheless, the vector competence of the larger adult females originating from larvae grown at the lower temperature was not modified compared to that of the control, while the females originating from exposed larvae grown at the higher temperature presented a significantly higher vector competence for the tested Sindbis virus (MRE16-strain) infection and dissemination rates than the control females [88]. The mechanism underlying the higher adult vector competence of these sublethally exposed larvae could not be assessed

in that study, but it was speculated that impairment of the immune system was involved in the increased vector competence in smaller adults [88].

Lingering damage to tissues—such as those of the midgut, as a result of larval or adult sublethal exposures to insecticides—is common in insects, including mosquitoes [81,89–91]. Because the insect midgut is a port of entry for most pathogens and is thus a barrier to be overcome before the pathogen can infect new hosts [92], any damage to the midgut or the peritrophic matrix (an extracellular matrix that surrounds the food bolus and is synthetized by the posterior midgut after a blood meal) could change a pathogen's ability to disseminate throughout the insect's body. *Bti*-based insecticides are normally used for larval control; upon ingestion, these insecticides damage midgut cells. Sublethal exposure to *Bti*-based products increases the susceptibility to dengue virus infections, but not to chikungunya in *Bti*-resistant *Ae. aegypti* [93]. It remains to be elucidated as to whether this is a common effect and related to the sublethal damage by *Bti* on the midgut, or is linked to genetic factors (since it was observed in a *Bti*-resistant mosquito population). On the other hand, the damaged midgut impairs the mosquito's digestion ability, inducing it to take smaller blood meals [81,89], which conversely reduces the likelihood of mosquitoes initially acquiring the pathogens.

Studies have been carried out considering the interaction between adult mosquitoes derived from exposed larvae and malaria-causing parasites [86,87,94]. For instance, the exposure of *Culex pipiens* to the neonicotinoid imidacloprid did not affect the life history traits of individuals nor the susceptibility of adults to infection by avian malaria parasite (*Plasmodium gallinaceum*) [86]. The exposure of larvae of *Cx. pipiens* to field-realistic doses of glyphosate, the most used herbicide worldwide, did not affect the individual survival, adult size, and female fecundity. Conversely, females derived from exposed larvae with the herbicide increased the probability of female infection by *Plasmodium relictum* [87]. The sublethal exposure to permethrin at larvae or adults of *An. gambiae* reduced the infection prevalence by *Plasmodium berghei* [94]. These studies with laboratory-consolidated models pointed that the level of interference of the exposure of the larvae to insecticides in the process of infection of mosquitoes depends on the species of both vector and pathogen and the mode of action of the compounds. Nevertheless, more realistic field studies are needed to better understand how larval exposure can interfere with the life cycle of pathogens in adult mosquitoes.

3. Changes in Pathogen Dissemination in Insecticide-Resistant Mosquitoes

Insecticides often do not reach 100% efficacy at controlling any given target species. Even when applied at recommended field rates, some compounds fail to reach the targeted insects because of biotic and abiotic factors associated with a surface covering failures, compound degradation, or runoff after heavy rainfalls [95,96]. Furthermore, it is not uncommon in any species that there are individuals capable of behaviorally avoiding such compounds or equipped with physiological tools capable of mitigating these compounds' actions. The result of these factors is that the applied insecticides will almost always leave some survivors. The individuals that survive such insecticide exposures by behavioral means or physiological mechanisms will then reproduce and transfer the traits that permitted their survival to their offspring, increasing the percentage of resistant individuals in the population across generations [32].

The two main classes of insecticide resistance mechanisms are alterations of target sites (e.g., the mutation in the voltage-gated sodium channel gene, for pyrethroids, or in the acetylcholinesterase gene, for organophosphates and carbamates), which generally reduces the binding rate of the insecticide and its target [19,97], and modifications of the insect's metabolism, which can occur via a variety of detoxification and excretion processes [98]. Both of these insecticide resistance mechanisms (i.e., target site modification and changes in metabolism) occur in mosquitoes. Furthermore, as already described for other insect groups [99–103], attention has recently been paid to behavioral resistance mechanisms to insecticides in mosquitoes [6,104,105]. The selection of resistance through any of these

mechanisms will shape the genetics of mosquito populations and could impact insect physiology and behavior, and therefore transmission abilities.

3.1. Target Site Insecticide Resistance

Most known cases of insecticide resistance related to target site alterations in mosquitoes involves knockdown resistance (*kdr*) mutations in the insect sodium channels, which are the major targets for the actions of pyrethroids [8,97,105,106]. The occurrence of *kdr* mutation, which reduces the action of insecticide molecules targeting voltage-gated sodium channels, was shown to also cause changes in the gating properties of such ionic channels [97,107–109]. Therefore, it is reasonable to expect modified firing activities (i.e., low action potential frequency) in some neural circuits of individuals carrying *kdr* mutations, potentially resulting in differential sensitivity to environmental cues.

Considering that a large part of a mosquito's host-seeking behaviors depends on olfactory sensory neurons [48,49], and that blood meal intake behavior has also been suggested to be modulated by specific sensory neurons in the tip of the mosquito stylet [110], modified neural activities in *kdr*-mutant mosquitoes could have important impacts on insect fitness. Diop et al. [111], for example, demonstrated that L1014F *kdr* homozygous *An. gambiae* had an impaired ability to locate holes in bed nets, suggesting a decrease in their overall sensory abilities due to this mutation. Another study also showed that an L1014F *kdr* insecticide-resistant strain of *An. gambiae* preferred hosts under insecticide-treated nets more than those under untreated nets, while isoline-susceptible mosquitoes did not discern between both netting options [112]. This preference for insecticide-protected hosts suggests that the L1014F *kdr* mutation in the voltage-gated sodium channels modulates the mosquitoes' host preference towards insecticide presence.

Differential susceptibility to pathogen infection has been reported in mosquitoes harboring *kdr* mutations, independently of insecticide exposure [113–115]. These studies indicated greater susceptibility of L1014F and L1014S *kdr* mutants of *An. gambiae* to infection by *P. falciparum*, which in turn represents a worsening scenario for malaria control in regions where pyrethroid insecticides are used heavily and mosquitoes have already evolved pyrethroid resistance [113–115]. Nonetheless, another study showed that even though L1014S *kdr* increased the susceptibility of mosquitoes to *P. falciparum* when pyrethroids were present, the insecticide had toxic effects directly on the pathogen, thus reducing the overall infection risk [116]. This suggests that the higher mosquito susceptibility to parasite infection is compensated for by the toxicity of the insecticide to the parasite itself.

When a mosquito harboring a given *kdr* mutation is selected, several other polymorphisms located in the vicinity of the voltage-gated sodium channel locus might also be selected for, even under weak selective pressure, and thus exponentially increase in the population [117]. These new frequencies of a certain haplotype might cause slight to strong changes in the mosquito's physiology (including its vector competence) or behavior. Previous investigations reported a positive correlation between high frequencies of a specific haplotype for the immune gene *ClipC9* and high frequencies of the L1014F *kdr* mutation, indicating that linked selection could be playing a role in shaping genetic traits other than the one under direct selective pressure [115]. When this immune gene was inhibited in the L1014F *kdr*-mutant *An. gambiae*, their susceptibility to *P. falciparum* increased, demonstrating the immune gene's direct role in controlling the mosquito's susceptibility to this pathogen. This immune gene was also shown to be located in a locus very close to the voltage-gated sodium channel gene [115]. This would, therefore, explain the multiple different outcomes of *kdr* mutations observed in several different populations, as these might be linked to different haplotypes of other genes that have indirect positive or negative impacts on the transmission rates, thus making the nature of these impacts hard to predict.

Another set of studies have also investigated a target-site mutation in the acetylcholinesterase gene (i.e., *Ace-1* G119S) that confers resistance to organophosphates and carbamates [22,118]. Given the involvement of this enzyme in the neural signaling, the same broad scenario of impacts of altered activity as results of mutations that were dis-

cussed for *kdr* resistance could be expected. However, at least one study provided evidence that the biochemical activity levels of this enzyme do not change with the mutation [119]. This is the only mutation found in this gene in both *Anopheles* and *Culex* and the selection process seemingly selected a single haplotype (*Ace-1*^{R}) with signals of linkage selections across at least two megabases in the genome of *An. gambiae* [120]. The reduced diversity across the genome of the resistant individuals therefore suggests a source for the several fitness costs and adult behavioral disadvantages reported by literature [120,121]. Although the specific pathways in which the selection for *Ace-1*^{R} allele can impact vector competence is not well known yet, its negative impact on fitness does not necessarily imply a lower transmission risk since higher *P. falciparum* infection prevalence was also described to occur in the resistant individuals compared with susceptible ones of same genetic background [122].

Insecticide resistance could also indirectly interfere with overall vectorial capacity, which is influenced by variables such as vector density and longevity as well as transmission of pathogens ([123] and references therein), by causing changes in insect fecundity, mainly through modulations to reproductive abilities by modifying mating or blood meal feeding abilities in resistant individuals. The previously mentioned changes in neural excitability may also directly impact the female-male communications and mating success of *kdr or Ace-1*^{R}-expressing individuals, or even impact the blood volumes that these females take to produce mature, viable eggs. Platt et al. [124] showed that a L1014F *kdr* mutation in *Anopheles* spp. benefitted mating competitiveness when in heterozygosis, but in homozygosis this mutation reduced mating competitiveness. Another study also showed the mating disadvantage for *An. gambiae* males carrying the *Ace-1*^{R} allele [121]. This observed effect on mating success could be caused by changes in neural olfactory perception of sexual aggregation pheromones, as previously demonstrated in *Ae. aegypti* [125], but further investigations are needed to confirm this hypothesis.

3.2. Metabolic Insecticide Resistance

Metabolic resistance in mosquito species usually involves increased expression of enzymes (e.g., esterase or monooxygenases dependent on cytochrome P450) associated with detoxification processes [64–67,126]. Unlike target-site mutations, the genetic mechanisms underlying metabolic resistance are more easily and logically linked to direct energy losses, since in most cases resistant insects possess higher expression levels of resistance enzymes, which surely consumes energy [73,127]. These energetic costs could indirectly or directly affect components of insect fitness (e.g., fecundity and longevity), pathogen transmission rates, and overall population densities [128,129]. Additionally, the regulatory mechanisms involved in the overexpression of detoxification genes could also select for other linked gene variants, as was discussed in the previous section [115].

The studies on the fitness costs of metabolic resistance in insect pests showed multiple different outcomes, which could be explained by differences in experimental designs or laboratory versus field conditions [41]. Nevertheless, the overexpression of both esterase and cytochrome P450 conferring resistance to two different insecticide groups (i.e., organophosphates, and pyrethroids) in *Cx. pipiens* reduced its energy reserves (e.g., glycogen, glucose, and lipids) by up to 30% [72,73]. In P450-overexpressing insects, smaller-bodied females and lower female emergence rates [72] were observed, which is suggestive of lower fecundity and potential population decreases, similarly to those demonstrated in organophosphate-resistant beetles overexpressing esterase enzymes [130]. These lower energy resources could also cause shorter adult lifespans, which could reduce the pathogen transmission risks, as was demonstrated for *Cx. pipiens* overexpressing esterase enzymes [131].

The effects of the overexpression of detoxification enzymes on the transmission rates of pathogens are still controversial. It has been suggested that esterase overproduction can interfere with *P. relictum* development in *Cx. pipiens* [132]. However, more recent studies have suggested that this resistance mechanism decreased the susceptibility of *Cx. pipiens* to

infection by this pathogen [133]. This reduction in infection rates more likely resulted from the increased expression of multiple immunity-related genes than from changes in energy costs [133]. This linked overexpression of multiple immune-related and detoxifying genes indicates that the same regulatory mechanisms and gene expression profiles are shared between these gene groups [117,133], suggesting that metabolic resistance can indirectly cause other physiological changes, including changes in vector competence.

The mechanisms involved in regulating the expression of detoxifying enzymes by the downregulation of microRNAs (i.e., short RNA sequences that bind to specific regions of their target mRNA to prevent protein synthesis) have recently been elucidated [134]. The downregulation of four of these post-transcriptional specific inhibitors of enzyme synthesis leads to higher levels of the cytochrome P450 enzymes that confer resistance to pyrethroids in *Cx. pipiens* [135,136]. These microRNAs are present in the genome in clusters, and their downregulation could impact several other physiological systems in the mosquitoes and interfere with their pathogen transmission abilities.

The overexpression of detoxifying enzymes might also interfere with mosquito host-seeking behaviors by disrupting their olfactory abilities. It is well-known in insects that for correct flight navigation towards an odorant source, every odorant molecule must be degraded after targeting its odorant receptor to allow the signal to be interrupted and new molecules recognized [137]. These processes are largely performed by enzymes, which are mainly esterase and cytochrome P450 enzymes [137,138]. Interestingly, members of a large class of cytochrome P450 genes (e.g., CYP3 and CYP4) that are strongly related to insecticide and other plant xenobiotic detoxification processes were also shown to be expressed at high levels in the antennae of herbivorous pests [138], suggesting a link between these two physiological functions (i.e., metabolism of insecticides and odorant compounds). Therefore, it is not known if high expression levels of esterase or cytochrome P450 enzymes could also metabolize odorant molecules faster in insecticide-resistant mosquitoes. The potential interference with host-seeking behavior in metabolically resistant mosquitoes would reduce their blood feeding frequency, fitness, and thus, pathogen transmission rates.

3.3. Behavioral Insecticide Resistance

The behavioral resistance discussed in this section relates to the failure of the insecticide to control the mosquito population because the mosquitoes are repelled by the insecticide and avoid exposure to it. This concept of behavioral resistance in mosquitoes has become a topic of concern due to the long-term, intensive use of IRS and LLIN over the last decades. Mosquitoes have recently been reported to show stronger repellent behaviors to pyrethroids than previously observed [6,33,139].

Since repellent behaviors to an insecticide directly change the exposure rate/time, their potential impacts on vectorial capacity through interactions with insecticides are very complex. Repellent behaviors can easily modulate the amount of insecticide to which an insect is exposed, therefore changing the dose it experiences from lethal to sublethal, and can also modulate the selection dynamics of physiological resistance to insecticides in different ways. We propose the following three main scenarios, considering the target site for the repellency is different than the one for lethality: (1) If a genotypic variant conferring insecticide repellence is already present in a population at a high frequency, this will decrease the likelihood of a physiological resistance mechanism being selected, since many susceptible individuals will survive exposure by being repelled. (2) If physiological resistance was selected before a repellent genotype arose in the population, then repellence is less likely to be selected since many resistant but non-repelled insects also survive. (3) Finally, when both variants are present in the population but not yet at high frequencies, they would reduce the selection pressure for each other, slowing down the selection for both genotypes, keeping their frequencies more stable until other factors disrupt such an equilibrium [32,140,141].

Transfluthrin, a pyrethroid, can cause behavioral repellency in susceptible *Ae. aegypti* adults by acting solely on the voltage-gated sodium channels. Transfluthrin-mediated

repellency is a likely result of insecticide actions in selected neuronal circuits [142]. Similar repellency mediated by the activation of sodium channels has also been demonstrated for natural pyrethrins [143]. The presence of the two *kdr* mutations (S989P and V1016G) in the sodium channel gene reduced pyrethroid-mediated repellency without impairing olfaction [143,144]. Other studies that used unrelated field-resistant populations have observed indirect effects of apparent linked or fitness-related alterations in repellency behavior to pyrethroids or other repellents in both *Ae. aegypti* and *An. gambiae* [144,145]. Nonetheless, further research is required on whether the observed increase in *Anopheles* repellency behavior to pyrethroids in the field is a direct or linked effect of *kdr* or metabolic pyrethroid resistance (with respect to lethality).

In addition to direct repellence behavior, the foraging period patterns of mosquitoes after the introduction of IRS and LLIN have also received special attention. Higher rates of mosquito bites in humans reported after the use of residual sprays and treated nets occurred through a temporal shift in mosquito foraging from late night to early evening (Figure 2) when people are still out of their beds or homes [139,146–149]. This shift could be a result of selections acting on trait variants and/or changes in ecological interaction among closely related species, especially in the case of the *An. gambiae sensu lato* species complex.

The existence of selective trait variants in a species leading to differences in endo/exophagy preferences was suggested in a study that detected changes in this foraging preference before and after the use of IRS and LLIN within a single species (i.e., *An. gambiae sensu stricto*) [150]. The behavioral trait that these selected phenotypes variants harbor (endophagy or exophagy) could be a result of single or multiple mutations in specific genes related to the circadian rhythms that influence time-specific behaviors, as has been observed in butterflies and drosophilids [151,152]. Additionally, changes in ecological interactions as a result of decreases in the density of one sibling species in the community that was lethally exposed to an insecticide, which benefitted and increased the prevalence and success of other species, were suggested by several studies. For instance, changes in species composition of *An. gambiae s.l.* occurred with a reduction in the density of more endophilic species (e.g., *An. gambiae s.s.*) and an increase in the density of more exophilic ones (e.g., *Anopheles arabiensis*) [147,153–155]. Similar shifts toward exophilic behavior at both the inter- and intraspecies levels have also been reported for the *Anopheles funestus* s.l. complex [147,156,157].

The above-mentioned ecological selectivity of insecticides for species with higher relative numbers of exophilic mosquitoes would benefit the adults of the exophilic species by reducing the overall larval density, and therefore, the competition stress experienced by its larvae. In turn, this could also cause an increase in the absolute number, size, and fitness of the adults of the remaining exophilic species [159]. The larger size of adults is related to there being longer-lived females in the population, which increases the pathogen transmission risks [31,160]. Therefore, the ecological selectivity of an insecticide, in addition to shaping the species assemblage towards individuals that are more exophilic or exophagic, may also induce higher mosquito fitness and pathogen transmission risks.

A higher prevalence of host-seeking in the early morning hours represents a greater risk of exposure of humans to biting mosquitoes [6,139,150]. Independently of the specific mechanism involved (e.g., inherited behavioral resistance, species assemblage changes, etc.), these behavioral changes could increase the pathogen transmission rates to similar or higher levels than target-site or metabolic insecticide resistance would, as has been demonstrated by two different mathematical models (i.e., Imperial and OpenMalaria models [161,162]) of malaria transmission. These two models predicted the effects of the increasing frequency of exophagy after the use of IRS and LLIN on mean entomological inoculation rates (infectious bites per person) (Figure 3) [6]. Therefore, more studies are needed to understand the very complex scenario resulting from the multiple species and multiple effects of the lethality of insecticides in these vectors in the real world.

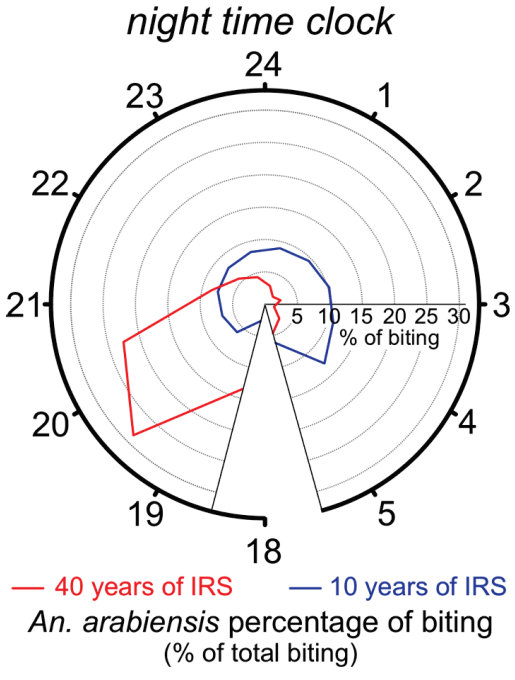

Figure 2. Time-related foraging behavioral changes potentially reducing the efficacy of *Anopheles* control. The colored lines within the clock represent the distribution of *An. arabiensis* biting times in places with short- (**blue**) or long-term (**red**) use of indoor residual sprays (IRS). The biting number decreases toward the center of the clock and increases toward the distal region of the clock. The clock indicates only the late afternoon and nighttime evaluation period from 6:30 p.m. (18:30) to 5:30 a.m. (5:30), as no data for other periods of the day were available. Adapted from Dukeen [158] and Yohannes [149].

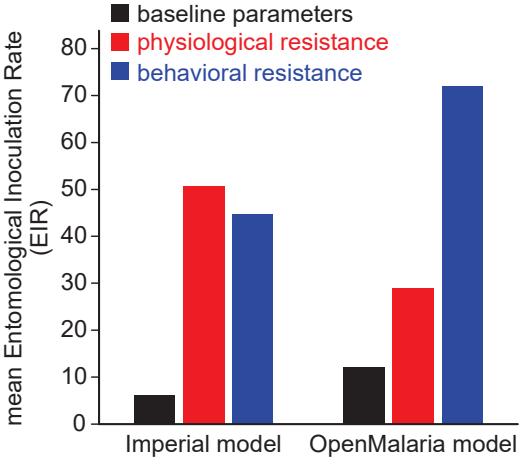

Figure 3. Malaria transmission model predictions as impacted by insecticide resistance after the use of LLIN and IRS. LLIN: long-lasting insecticidal nets. IRS: indoors residual spray. EIR: entomological inoculation rate (infectious bites per person). Adapted from Gatton et al. [6].

4. Conclusions

A broader understanding of the effects of insecticides on vectors of human pathogens is needed to support continuous efforts aimed at epidemic reductions. Every year, new disease outbreaks occur, such as the Zika epidemic outbreak in 2015 in South America and the increasing number of cases of Dengue infection worldwide. These outbreaks point out the fact that, despite causing high mosquito mortality, the sublethal effects mediated by insecticides on the behaviors and physiology of mosquitoes can also influence their transmission of pathogens. A large number of studies have suggested that insecticide resistance has modified the physiology, blood-feeding behavior, and reproduction of mosquitoes, and to some extent the dynamics of many diseases that vector mosquitoes can spread. The present comprehensive review and discussion on how unintentional selection for insecticide resistance can drive the overall transmission risks of pathogens by different heritable traits and mechanisms in mosquitoes could help us to better predict, understand, and mitigate common and unexpected epidemics like those that have occurred recently.

The effects of sublethal exposures, on the other hand, involve even more dynamic environmental and ecological interactions that are much less tractable and reproducible by studies when compared with the effects of insecticide resistance in terms of population genetic changes. Thus, even though numerous studies have been done on these sublethal effects, establishing comprehensive and predictable links between the effects of sublethal exposures and changes in vector competence is still a challenge. Therefore, the sublethal effects of insecticides on mosquito vector competence might still be considered a large research gap, with there being a long way to go before we can obtain a more comprehensive understanding of their effects and mechanisms.

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Review

Review and Meta-Analysis of the Evidence for Choosing between Specific Pyrethroids for Programmatic Purposes

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Simple Summary: A group of insecticides, called pyrethroids, are the main strategy for controlling the mosquito vectors of malaria. Pyrethroids are used in all insecticide-treated bednets, and many indoor residual spray programmes (in which insecticides are sprayed on the interior walls of houses). There are different types of pyrethroids within the class (e.g., deltamethrin and permethrin). Across the world, mosquitoes are showing signs of resistance to the pyrethroids, such as reduced mortality following contact. However, it is unclear if this resistance is uniform across the pyrethroid class (i.e., if a mosquito is resistant to deltamethrin, whether it is resistant to permethrin at the same level). In addition, it is not known if switching between different pyrethroids can be used to effectively maintain mosquito control when resistance to a single pyrethroid has been detected. This review examined the evidence from molecular studies, resistance testing from laboratory and field data, and mosquito behavioural assays to answer these questions. The evidence suggested that in areas where pyrethroid resistance exists, different mortality seen between the pyrethroids is not necessarily indicative of an operationally relevant difference in control performance, and there is no reason to rotate between common pyrethroids (i.e., deltamethrin, permethrin, and alpha-cypermethrin) as an insecticide resistance management strategy.

Abstract: Pyrethroid resistance is widespread in malaria vectors. However, differential mortality in discriminating dose assays to different pyrethroids is often observed in wild populations. When this occurs, it is unclear if this differential mortality should be interpreted as an indication of differential levels of susceptibility within the pyrethroid class, and if so, if countries should consider selecting one specific pyrethroid for programmatic use over another. A review of evidence from molecular studies, resistance testing with laboratory colonies and wild populations, and mosquito behavioural assays were conducted to answer these questions. Evidence suggested that in areas where pyrethroid resistance exists, different results in insecticide susceptibility assays with specific pyrethroids currently in common use (deltamethrin, permethrin, α-cypermethrin, and λ-cyhalothrin) are not necessarily indicative of an operationally relevant difference in potential performance. Consequently, it is not advisable to use rotation between these pyrethroids as an insecticide-resistance management strategy. Less commonly used pyrethroids (bifenthrin and etofenprox) may have sufficiently different modes of action, though further work is needed to examine how this may apply to insecticide resistance management.

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1. Introduction

Pyrethroids are present in all WHO-prequalified insecticide-treated nets (ITNs), and are also used for indoor residual spraying (IRS) [1]. Pyrethroid resistance is widespread in malaria vectors [2,3], and differential mortality in discriminating dose bioassays between pyrethroids is often observed during susceptibility monitoring in lab strains and in wild populations. There is uncertainty about whether current methods for monitoring insecticide resistance can reliably identify moderately different levels of resistance within the pyrethroid class. When differential mortality is observed in discriminating dose bioassays, it is unclear if this should be interpreted as an indication of differential levels of susceptibility within the pyrethroid class, or if this could arise due to inherent variability in bioassay results or differently calibrated discriminating doses within the pyrethroid class. Considering this, when differential susceptibility is observed, there is a question regarding whether countries can use targeted or preferential use of specific pyrethroid insecticides as an effective resistance management strategy. This is important, as maintaining the efficacy of pyrethroids is vital to malaria control while we wait for novel active ingredients (AIs) with new modes of action (MoA) to be developed. To address these questions, this review examined evidence from molecular studies, insecticide resistance patterns and bioassay results from laboratory colonies and field populations, and lessons from behavioural assays.

2. Do Discriminating Doses Accurately Detect Resistance in Different Pyrethroids?

Current methods for monitoring insecticide resistance are based on classifying phenotypic resistance, which is typically measured using standardised tests, such as WHO susceptibility bioassays [4] and CDC bottle assays [5]. These tests expose mosquito populations (wild-collected females or those reared from collected larvae) to predefined "discriminating doses" (DDs) of an insecticide, and record mosquito knockdown and mortality at defined times postexposure. A DD is defined by WHO [4] as "a concentration of an insecticide that, in a standard period of exposure, is used to discriminate the proportions of susceptible and resistant phenotypes in a sample of a mosquito population". It is calculated by establishing a dose response in susceptible mosquitoes, and then calculating either "twice the lowest concentration that gave systematically 100% mortality (i.e., LC_{100})" or "twice the LC_{99} values" estimated from this baseline susceptibility testing. Under- or overestimation of discriminating doses could have an impact on the accurate detection of insecticide resistance in wild populations, and misclassification of lab strains.

In 2016, following increasing evidence of the limitations of discriminating dose assays [6,7], the WHO updated their monitoring guidance to include additional testing of resistant populations at 5× and 10× DDs to provide further information on the intensity or "strength" of phenotypic resistance [4].

The current WHO DDs for *Anopheles* for deltamethrin (0.05%) and permethrin (0.75%) were established, along with other pyrethroids, through an international multicentre study in 1998 [8]. Some other pyrethroids, including α-cypermethrin, were not included in this original study; the α-cypermethrin recommended DD (0.05%) is tentative, and currently under validation by the WHO in a new multicentre study [9]. The 1998 study exposed known insecticide-"susceptible" strains of *Anopheles albimanus*, *Anopheles gambiae*, and *Anopheles stephensi* to up to five different concentrations of each insecticide using WHO tube bioassays. Mortality postexposure was then analysed using Probit regression to establish a single lethal dose for all *Anopheles* species for each compound, which was doubled to give the DDs that are still recommended today.

In this review, we reanalysed the publicly available data from the 1998 study to establish if the recommended DDs are suitable and comparable. Using these data, we were unable to establish the LC_{100} for permethrin, since in one centre some mosquitoes survived the highest concentration tested (Mali, *An. gambiae*, Mopti strain, 1% permethrin, 99.2% mortality). For deltamethrin, data were incomplete (0.1% deltamethrin killed 100% of the exposed mosquitoes, but not all centres tested the 0.1% concentration against all strains).

We then used Probit analysis (PoloJR program within PoloSuite, Version 2.1) to obtain LD_{99} values. The DDs calculated from this when all the data were pooled were 0.1% for deltamethrin and 1.46% for permethrin (Figure 1, Table 1), around double the final DDs recommended by the 1998 multicentre study [8]. The concentrations tested in the original study did not produce a full range of mortality (i.e., 0–100%), which resulted in poorly fitting dose-response curves. Poorly fitting dose-response curves were also observed when strains and species were pooled separately (Supplementary Material, Figure S1). In several cases, our Probit analysis could not calculate the lethal concentration for individual sites/strains or estimate meaningful confidence intervals around the LC values (Supplementary Material, Table S1). Robust dose-response relationships were not observed, and in some study sites, mortality was never below 80% in the strain tested. The original selection of DDs was thus not well supported by the data.

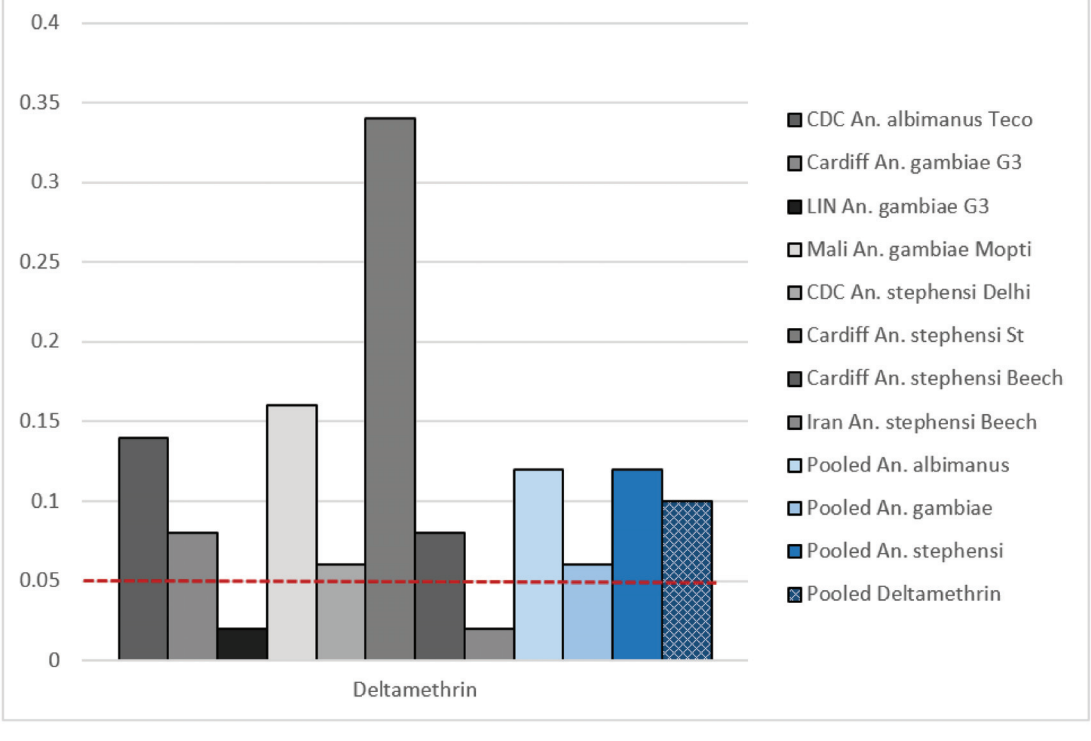

Figure 1. *Cont.*

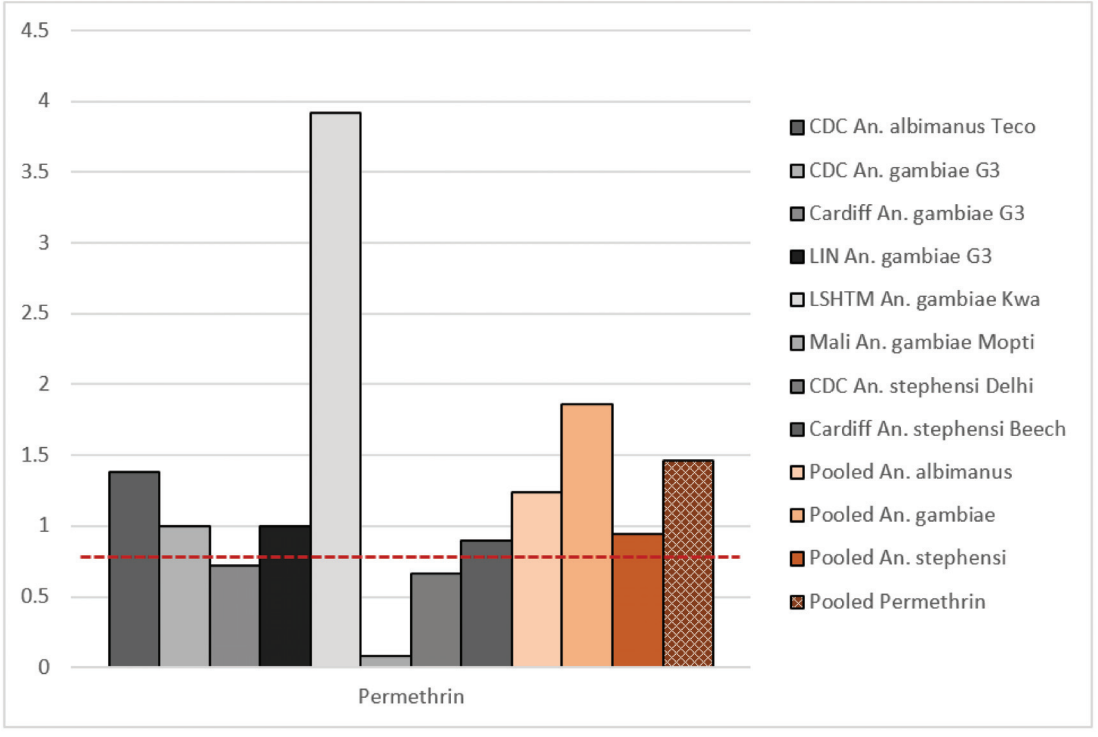

Figure 1. Calculated discriminating doses (%) for deltamethrin (**top**) and permethrin (**bottom**). Points show individual site/strain combinations, and data pooled by species or overall and by the insecticide. Site/strain combinations testing <3 concentrations of an insecticide and datasets that were not robust enough to calculate lethal dose matrixes were excluded. Discriminating doses were set at 2× the calculated lethal dose at which 99% (LD_{99}) of test mosquitoes were killed. The dashed red line represents the current WHO-recommended DD (0.05% deltamethrin and 0.75% permethrin). LSHTM *An. stephensi* data are omitted here to improve visualisation of other data points, as their calculated DDs were high. A version with this included can be found in Supplementary Material, Figure S2. Bar charts displaying mosquito mortality (%) following exposure to permethrin and deltamethrin in individual WHO tube bioassays can be found in the Supplementary Material (Figures S3 and S4).

Table 1. Probit analysis of 1998 WHO multicentre study. The discriminating dose is 2× the LD_{99}. Abbreviations: LD = lethal dose; CI = confidence interval; DD = discriminating dose; DoF = degrees of freedom.

	Deltamethrin	**Permethrin**
Number exposed	8258	9582
LD_{99} (95% CI)	0.05 (0.023–1.166)	0.73 (0.452–2.067)
Calculated DD	0.1	1.46
Chi-square [1]	2215.41	2055.49
Heterogeneity (DoF)	39.56 (56)	32.12 (64)
Current WHO DD	0.05	0.75

[1] Chi-square provides a measure of fit.

Most centres within the study appeared to have diverged from the common protocol in terms of sample size and replicates tested. In the original protocol, 2–3 replicates of 100 mosquitoes (200–300 mosquitoes) should have been tested per insecticide concentration. However, at some study sites, $n < 25$ mosquitoes per concentration were tested. Mosquitoes were tested at 1—3 days old, whereas current guidelines state 3–5-day-old mosquitoes

should be used [4]. This may have influenced results, particularly in 1-day-olds, as their cuticles may not have hardened [10].

In the original report, there are a lack of raw data, and it is not clear which data were used in proposing the final doses. The report states that more weight was given to studies in which mortalities were clustered around similar values; however, which analyses were weighted or the methodology for weighting was not specified. It is therefore not possible to establish why the WHO-recommended DDs differed from the ones calculated in this review. Given the unclear rationale for the DDs recommended for permethrin and deltamethrin, and that the data used to calculate them were unclear, their comparability is questionable. The DDs of these pyrethroids were not calibrated against one another, and the assay was not designed to compare compounds, but to monitor for resistance to each independently. This was a challenge when trying to draw reliable conclusions about the relative efficacy of, or resistance to, these two pyrethroids based on data collected using these DDs.

3. Is there Molecular Evidence for Differential Resistance among Members of the Pyrethroid Insecticide Class?

Molecular studies indicate that structurally diverse pyrethroids such as tefluthrin, transfluthrin, bifenthrin, and etofenprox, which lack the common structural moiety of most pyrethroids, may interact differently with the common resistance mechanisms found in insect populations [11–13] (Figure 2). To assess cross-resistance within the pyrethroids in terms of their interactions with key cytochrome P450 enzymes (hereafter P450s) and resistance in vector populations, P450 functional activity data with pyrethroids were compared with field mortality data [12]. Figure 3 shows the relationships among pyrethroids in terms of their binding affinity to and depletion by key P450 enzymes known to confer metabolic pyrethroid resistance in *Anopheles gambiae* (s.l.) in comparison to mortality data among pyrethroids. Bifenthrin diverges from the pyrethroids commonly used in malaria vector control in terms of binding affinity to, and depletion by, P450s from African *Anopheles* species, while etofenprox diverges from the other pyrethroids in terms of binding affinity to these P450s, but not depletion by them [12]. When these relative differences found by molecular studies were compared to relative differences in the prevalence of resistance to each pyrethroid within African malaria vector populations, the potential divergence of etofenprox was observed in both the molecular studies and the field studies, but bifenthrin has not yet been tested in field studies of African malaria vectors (Figure 3).

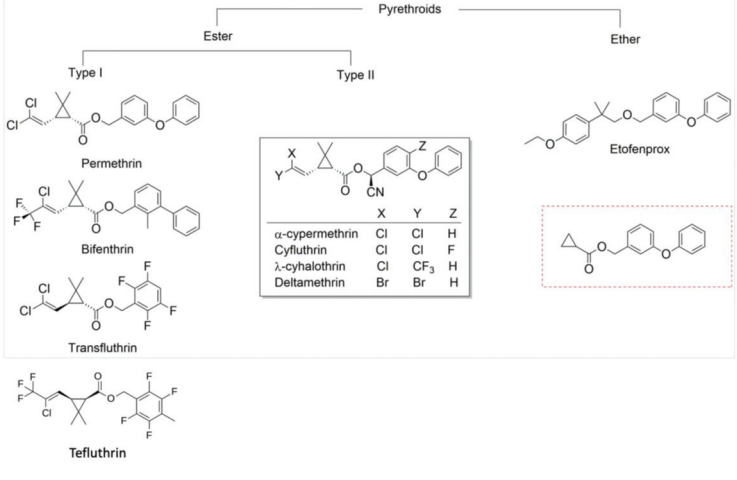

Figure 2. Chemical structure of pyrethroid insecticides used for malaria vector control. The common scaffold of pyrethroids, boxed in red, was identified by searching 230 million compounds available in the ZINC database (https://zinc.docking.org, accessed on 23 February 2020). Adapted from [12].

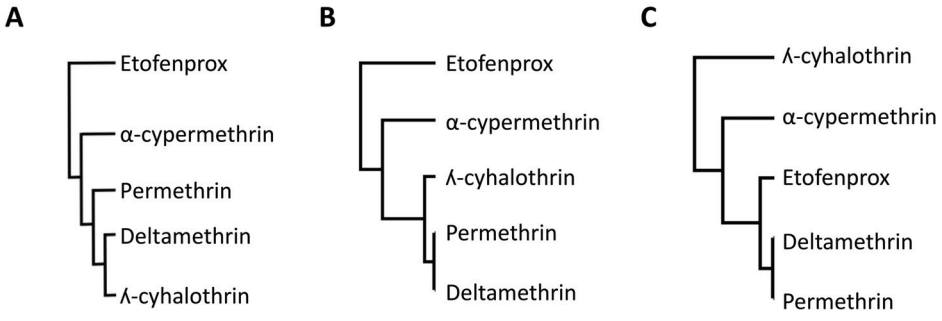

Figure 3. Hierarchical relationships among pyrethroids defined using data on resistance in vectors and functional activity data. The dendrograms were constructed using correlations in mortality across African malaria vector populations (Pearson's correlation coefficient) (**A**), binding affinity values (IC50), (**B**) and insecticide depletion values (%) (**C**). Adapted from [12].

4. What Intrinsic Variability Do We See from Dose-Response Assays in the Lab?

Discriminating dose bioassays are routinely used to detect and monitor insecticide resistance in mosquito populations. When conducted in well-controlled lab settings, factors such as temperature, humidity, and mosquito rearing are standardised to minimise their effects on mosquito mortality. Examining repeated measurements taken in these settings, with as many variables as possible controlled, allowed us to investigate what intrinsic variability stemmed from the assay itself.

In this review, we collated and analysed discriminating dose data from the Liverpool Insecticide Testing Establishment (LITE) and Vector Biology Department at the Liverpool School of Tropical Medicine (LSTM). The *Anopheles* mosquito colonies maintained by each group are profiled at least annually using standard WHO susceptibility tests. Additionally, each group applies deltamethrin selection to resistant strains every 3–5 generations to maintain pyrethroid resistance (i.e., mosquitoes are exposed to deltamethrin using standardised procedures, and survivors are used to maintain the colony). In most instances, this selection follows the same protocol as the WHO susceptibility test (exposure to 0.05% deltamethrin for 1 h). Additionally, when testing novel or repurposed chemistries, a positive pyrethroid control is often used in experiments—overall, these studies represent a set of repeated bioassay (tube or bottle) measurements under uniform testing conditions and using the same mosquito colonies.

For each mosquito strain, the mortality data for profiling, selection, or other experiments were compiled. When colonies of a strain were held in both LITE and LSTM, these data were considered separately. For each strain/insecticide combination, summary statistics of mortality were calculated (the range, interquartile range, mean, median, variance, and standard deviation). A Welch's *t*-test was used to compare mean mosquito mortality following exposure to different pyrethroids, or the same pyrethroid in different assays. The analysis was conducted with R statistical software version 3.6.2 (12 December 2019) [14].

In general, following exposure of characterised lab strains in WHO tube bioassays under controlled conditions, the level of variability in mortality among test replicates exposed to a single compound was greater in moderately resistant strains (mean mortality > 15%) (Figure 4). In this example, the standard deviation for FUMOZ-R (mean mortality 25.24%) following exposure to permethrin was 29.12, and for Tiassalé 13 (mean mortality 15.97%) it was 16.01. In instances in which insecticide/strain replicate numbers were lower (<10 replicates in some cases), variability was lower (Supplementary Material, Table S2 and Figure S5). Boxplots summarising mortality of all strains examined to all insecticides can be found in Supplementary Material, Figures S6–S7.

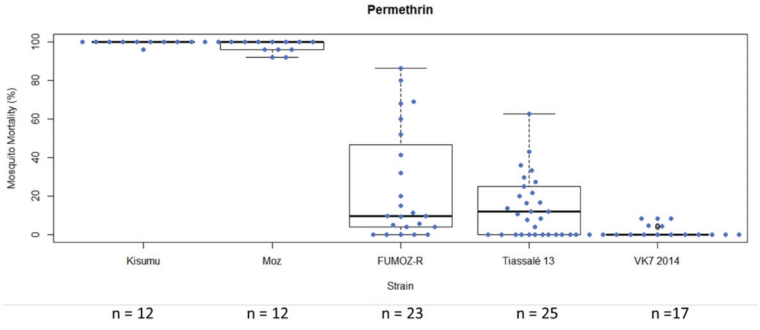

Figure 4. Box plot summarising mosquito mortality following exposure to permethrin 0.75% in a standard WHO tube bioassay in LITE strains. Each box represents a different mosquito strain. Coloured circles and n values indicate each tube replicate (not total mosquito numbers).

In CDC bottle assays (with 0.00125 µg/bottle permethrin or 0.00125 µg/bottle permethrin + 400 µg/bottle piperonyl butoxide (PBO) simultaneously [15]), greater variability in mortality was again observed in moderately resistant strains (mean mortality 15–80%) compared to highly resistant (mean mortality < 15%) or more susceptible strains (mean mortality > 80%) (Figure 5). This was mirrored in the PBO treatments in which mortality was greatly increased in the resistant strains and the variability generally decreased (though there was still considerable heterogeneity in the more resistant strains). Further investigation is required to establish the inherent variability in PBO synergism assays, relative to DD bioassays.

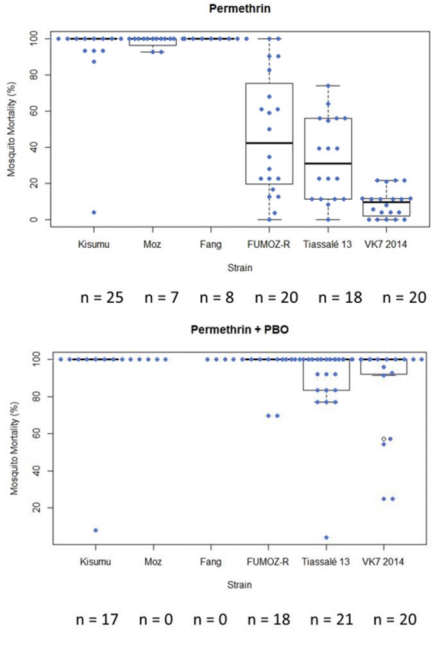

Figure 5. Box plot summarising mosquito mortality following exposure to permethrin 20 µg /bottle (**top**), or permethrin 20 µg/bottle + PBO (**bottom**) in a standard CDC bottle bioassay. Each box represents a different mosquito strain. Strains here are those maintained by LITE at LSTM. Coloured circles and n values indicate each replicate bottle.

When comparing the two testing methods in resistant strains, both mean mortality and variability (standard deviation) in mortality were greater in the CDC bottle bioassay compared to the WHO tube test (in response to their respective discriminating doses), but comparable in susceptible strains (in which almost all mosquitoes died; Figure 6). Previous studies have reported variability in comparability between WHO and CDC bioassay results [16], suggesting reasonable interchangeability in identifying susceptible populations, but less so when substantial resistance is present [7]. Dose-response experiments are perhaps more easily performed using bottles, but very high concentrations may prove difficult due to issues with solubility or crystallization of active ingredients [17].

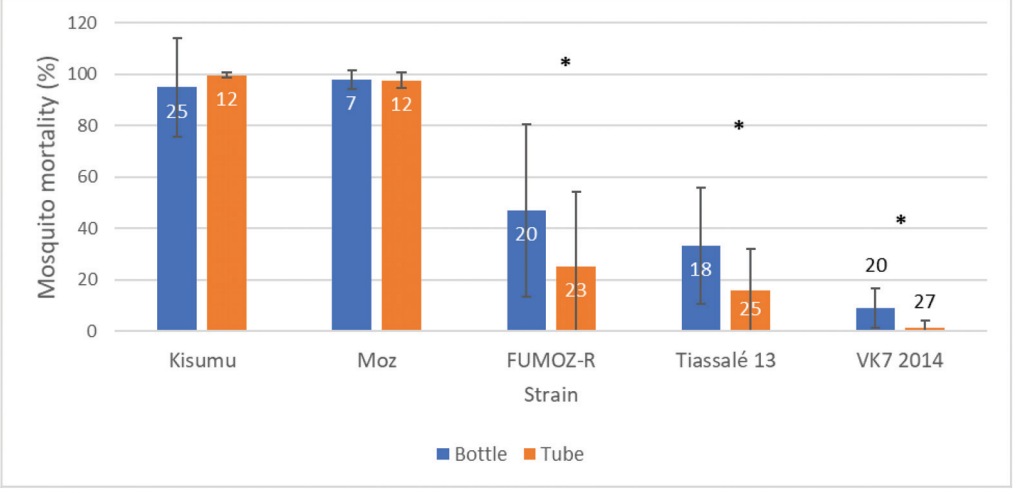

Figure 6. Average mosquito mortality following exposure to discriminating doses of permethrin in CDC bottles (0.00125 µg/bottle) (blue bars) or WHO tube (0.75%) bioassays (orange bars) in LITE strains. Numbers above or in bars indicate the number of replicate bottles or tubes. Error bars show the standard deviation to indicate variability between replicates. Asterisks above bars indicate where mean mortalities were significantly different ($p < 0.05$, Welch's *t*-test).

5. Is There Evidence for Divergent Resistance in Lab Colonies Routinely Selected Using a Single Pyrethroid?

Comparing mortality in the same laboratory populations routinely tested against DDs of different pyrethroids allowed for a comparison of whether susceptibility within any of the strains differed between compounds, and whether this changed over time (albeit an imperfect comparison, given the uncertainty around the DDs themselves and the level of variability in assay results already described). In general, susceptibility to permethrin, deltamethrin, and α-cypermethrin was similar within individual strains (Figure 7).

The Tiassalé 13 strain maintained by LSTM exhibited, on average, higher mortality against deltamethrin than to other pyrethroids, though no difference was seen in the Tiassalé 13 strain in experiments conducted at LITE. The differences in mortality between the insecticides was less than that seen in repeated experiments within the same strain (Figure 7), making it difficult to conclude if there were true differences in susceptibility between different pyrethroids between strains based on these data. The laboratory strains tested in this dataset had been selected with deltamethrin for up to 6 years [18]. In the absence of selection against all pyrethroids, we could expect divergence over time if differences existed between the pyrethroids, yet there was no obvious trend towards increasing relative resistance to deltamethrin. When mortality in the WHO tube bioassay was plotted over time, no obvious trends or changes in mortality from year to year across all strains were detected, and any temporal changes within strains seemed consistent across all pyrethroids tested (Supplementary Material Figure S5). These data were collected

passively during routine monitoring of the resistance profiles of reference colonies, and a targeted investigation into the effects of selection pressure on differential resistance to individual pyrethroids is needed to reach a more robust conclusion.

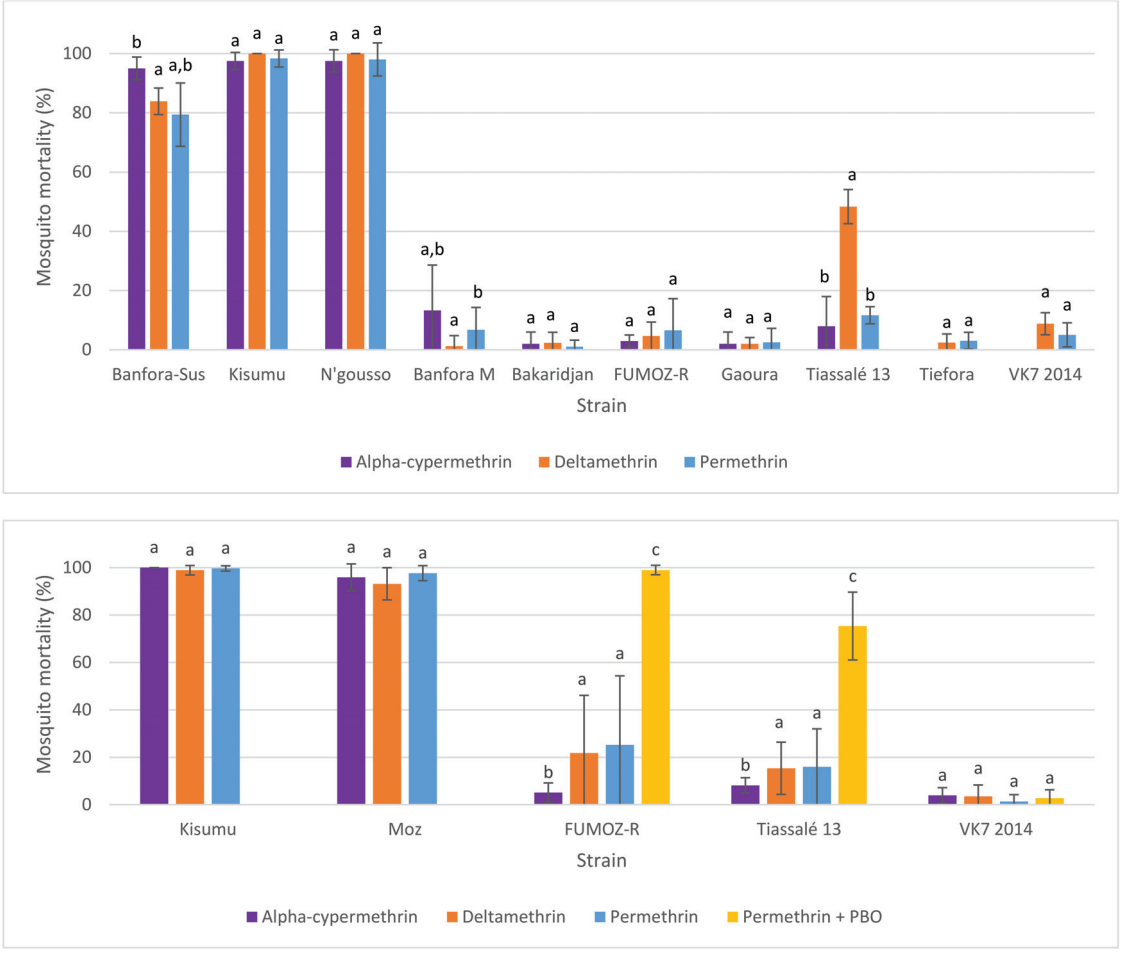

Figure 7. Average mosquito mortality following exposure to α-cypermethrin 0.05% (purple), deltamethrin 0.05% (orange), permethrin 0.75% (blue), or permethrin 0.75% preceded by piperonyl butoxide (PBO) (yellow) in a standard WHO tube bioassay in LSTM (**top**) or LITE (**bottom**) strains. Bars sharing the same superscript letter were not significantly different ($p < 0.05$, Welch's *t*-test). Error bars show the standard deviation to indicate variability between replicates. The *p*-values are shown in Supplementary Material, Table S3.

6. What Are Potential Sources of (Non-Resistance-Associated) Variability in the Discriminating Dose Bioassay?

The WHO [4] gives precise parameters for some of the key environmental conditions that should be established when carrying out bioassays. Poor larval rearing conditions (e.g., crowding and/or low food) can have extreme effects on bioassay results [19], but these are relatively easy to control under standard insectary conditions. Nevertheless, details of the rearing conditions employed are often scant in reports of bioassay data, and expanded descriptions would help to assess whether this may be an important source of variability. Time-of-day effects on bioassay results do not seem to be well-explored in the literature, but

circadian rhythmicity of many detoxification genes suggest that mosquitoes tested at night may not show the same resistance patterns as those tested during the day [20,21]. This may be significant for the operational interpretation of results, considering that African *Anopheles* typically bite at night. However, this is unlikely to be a major source of variability affecting bioassay data, since tests are typically performed during daytime hours. Nevertheless, reporting of testing times along with bioassay results would be a good practice to adopt more widely.

In contrast, under field conditions, WHO-specified temperature and relative humidity are often difficult to achieve and maintain, and the effects of variation can be highly significant. As part of a genomewide association study with sampling and testing conducted in a field insectary in Uganda that lacked environmental controls, Weetman et al. (2018) detected a strong and highly statistically significant decline in *An. gambiae* mortality as humidity increased (Figure 8A). In this study, temperature also varied, but did not independently account for the statistically significant variation in mortality. In the WHO-IIR (impacts of insecticide resistance multicentre trial), temporal repeatability of results from sentinel sites in Sudan was poor [22], and a significant contributory factor may have been variability in temperature and relative humidity, which correlated strongly (Figure 8B). As temperature and humidity decreased, mortality increased at the discriminating dose in pyrethroid bioassays with the *An. arabiensis* tested. Interestingly, this was the opposite directionality to that observed in Ugandan *An. gambiae*, and may reflect the differences in aridity tolerance between the species [23]. Significant, but inconsistent, effects of temperature on bioassay mortality have also been reported among laboratory colonies of *An. stephensi* [24], *An. arabiensis*, and *An. funestus* [25]. Whether or not the contrast in the direction of effects of humidity and temperature between studies reflects differential physiological adaptations of the species studied, such variability highlights the difficulty in predicting and statistically controlling for temperature and humidity effects. Indeed, these may depend quantitatively on the humidity–temperature optimum-tolerance profiles of the population tested. Nevertheless, studies should record and report these variables accurately, so that caveats can be applied when concluding datasets obtained under differing ambient conditions.

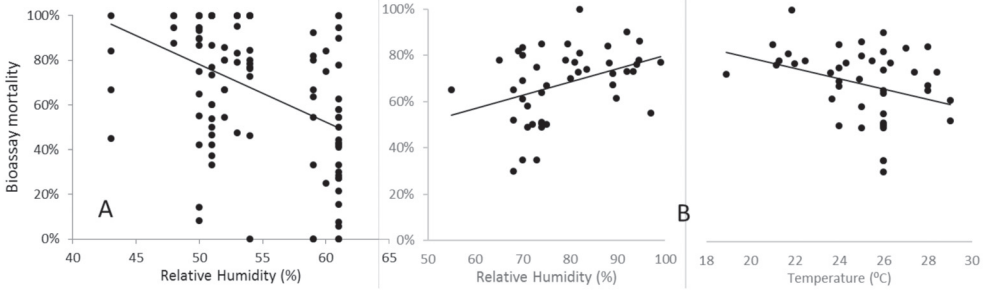

Figure 8. Effects of environmental conditions recorded in field insectaries during the insecticide exposure period on bioassay mortality (**A**) humidity on permethrin assays performed on Ugandan *An. gambiae* [26] and (**B**) humidity and temperature on deltamethrin assays on Sudanese *An. arabiensis* [22]. All regression lines were highly significant.

The age of mosquitoes tested is also an important consideration, and multiple studies have shown that mortality in pyrethroid bioassays performed on *An. gambiae*, *An. coluzzii*, and *An. arabiensis* increases with mosquito age [27–31]. However, this pattern may not be universally true across insecticides and resistance mechanisms. Recent work on pirimiphos-methyl-resistant *An. gambiae* from Ghana, in which resistance is strongly determined by combinations of target site mutations, showed no differential trend in mortality over ages spanning 3–15 days [32]. However, provided mosquitoes were reared in the laboratory from larvae or eggs, we are not aware of any results from bioassays that showed decreases in mortality with age. With a preference to test the least-susceptible age

group in insecticide bioassays, this argues for the current approach of targeting young (but at least 2-day-old) adults.

Physiological conditions of females, not directly related to age, may play a less predictable role in variation in bioassay mortality. The effect of blood feeding has been primarily studied in laboratory strains of *An. arabiensis* or *An. funestus*, in which a moderate and transient reduction in permethrin and deltamethrin mortality after a single blood meal was detected [33,34]. These findings have recently been replicated with field-collected samples of *An. gambiae* from Kenya [31]. The proposed mechanism for this is the upregulation of a vast number of detoxification genes in response to the oxidative stress caused by the intake of blood by female mosquitoes [35]. The magnitude of effect appeared to be dramatically greater if multiple blood meals were taken before insecticide exposure (up to 60% reduction in mortality for permethrin and deltamethrin, even in 21-day-old females) [34]. Further studies on the same strains showed that multiple bloodmeals appeared to be linked to a sustained enhancement in the ability to defend against oxidative stress, a common toxic effect of pyrethroid exposure [36]. Repeated sublethal prior insecticide exposures might have a similar effect, but results to date are inconclusive [27], possibly because of the conflicting effects of priming via enzyme induction from insecticide pre-exposure, and delayed effects of sublethal exposures on mortality [37]. In the absence of additional studies, the ubiquity and magnitude of the effects of repeated sublethal insecticide exposure, and more concerningly, repeated blood feeding, are difficult to predict, but suggest that in combination with the more estimable age effects, performing bioassays on adult females caught directly from the wild may provide highly variable or even biased results.

A common feature of most published works describing bioassay data is a relatively poor description of the sampling methodology, which is usually performed following an opportunistic plan. Generally, few details are provided to describe the range of collection sites, and often only a single GPS location is given, which can probably be assumed to represent an approximate central point for sites contained within a polygon of unknown size [38]. For comparative studies involving bioassay data, this is problematic because: (a) chances of repeatability are lowered by lack of collection detail, and (b) samples may lack independence as biological replicates, which may introduce bias or inflate statistical power. A priori, the predicted magnitude of this effect is expected to depend on the collection method employed. If adults are collected, they may be either tested directly (noting the inherent problems with testing adults with unknown variation in physiological status and age described above) or used to obtain eggs, which may be combined and reared for adult bioassays. Collected adults would typically be assumed not to be closely related; whilst if their eggs are used, the level of relatedness in the resultant sample would be expected to be roughly proportional to the number of families combined (assuming equal contributions from each). However, for the *An. gambiae* complex, the most common method of obtaining samples involves collecting larvae from larval habitats, presenting a potentially significant, but unknown, likelihood of sampling siblings. A strategy of collecting from as many local larval habitats as possible might reasonably be expected to ameliorate this problem to some extent. Yet, to our knowledge, there has been no previous study examining relatedness levels in collections made following any of the above collection strategies. As part of genomewide association studies using bioassay-based insecticide-resistance phenotypes, larval samples were collected from Yaoundé, Cameroon, and Dodowa, Ghana in 2006 [39], and adults from Tororo, Uganda in 2008, from which offspring were obtained for bioassay testing [23]. Further samples were obtained from recently and long-established colonies at LSTM, and all samples were genotyped using a custom Illumina array. More recent collections were made from over 50 locations (each represented by several larval habitats within a radius of a maximum of a few kilometres, and often much less) across southern Ghana in 2016. Genomes of a random sample from each collection were sequenced at low coverage [32]. In each dataset, relatedness categories among the samples were estimated (Figure 9).

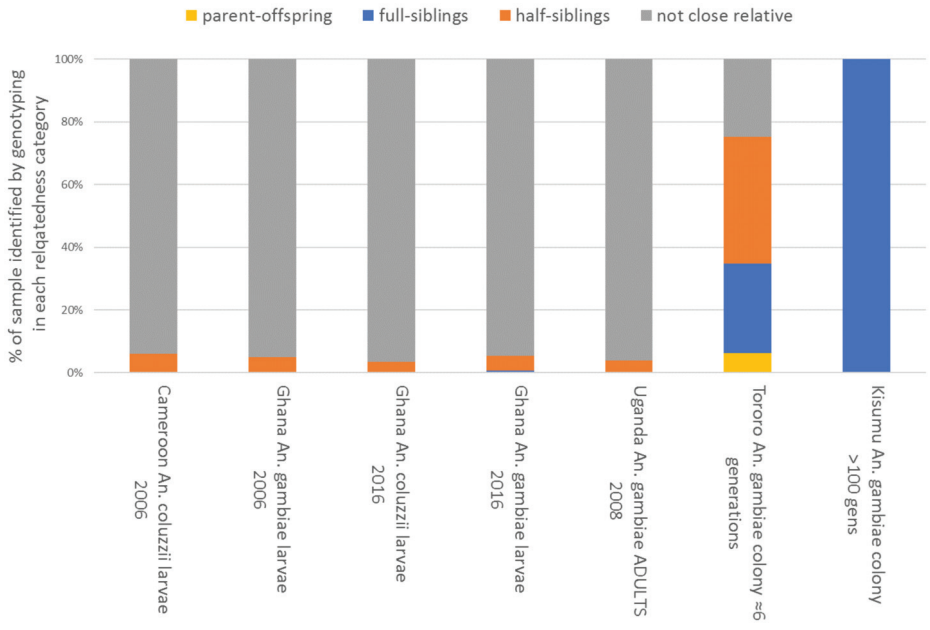

Figure 9. Genotype-based identification of close relatives in female samples collected as larvae from nearby collection locations in 2006 and 2016, and as adults in 2008; samples from both a recently established and a very long term colony are also shown for comparison [40]. Results from 2016 were estimated from data on ≈160 samples for each species at 2229 chromosome 3 SNP markers (see Supplementary Material, Figure S8). All other data were from 286 chromosomes 3 SNP markers (37) with field sample sizes of ≈180 for adults and 600–700 for larvae. Relationship categories were estimated using ML-Relate [41].

Results proved to be surprising. Larval collections in 2006 contained only approximately 5% of siblings, and those from within the same locations (i.e., sets of local larval habitats) in the 2016 collections showed a similar overall average, though occasionally sites showed much higher values (maximum = 46% related as half- or full siblings; see Supplementary Material, Figure S8). This suggests that relatedness within larval habitats is much lower than might typically be assumed, and samples dominated by siblings are probably the exception, rather than the norm, provided efforts are made to sample as many locally accessible sites as possible. This is concordant with recent results from *An. arabiensis* showing that productive larval habitats contained many larvae because they contained many families, rather than large numbers from single or few families [42]. Relatedness among the adults collected from houses in Uganda was similar to that among the larval collections, and, as expected, all the estimates from field sampling contrasted very markedly with the majority of close relatives seen in the recently established colony, and especially the long-established Kisumu strain. Overall, these results suggested that with reasonable diligence, most larval samples of *An. gambiae* might be assumed as broadly unrelated in locations where multiple larval habitats are available, providing little problem with the assumptions of independence for statistical models. When obtaining larvae is difficult, obtaining eggs from many females presents a reasonable alternative, including reporting details of the number of egg batches combined alongside data.

A final consideration in sampling is species identification. Failure to differentiate morphologically cryptic species within complexes or groups can create significant biases when comparing results among studies. When relative species composition varies in space or time, failure to identify which are being tested can lead to misinterpretation of causality if insecticide resistance differs interspecifically, which is often the case for *An. gambiae*

or *An. coluzzii* vs. other species complex members [43]. Multiple, cheap, and reliable molecular assays are available to identify species, and their application is crucial, though initial morphological identification to the level of species complex or group is always strongly advised [44].

7. What Is the Evidence for the Existence of Divergent Resistance between Pyrethroids? Can Differences Seen in Molecular Studies (Section 3) Be Detected in Wild Mosquito Populations?

The WHO intensity bioassay [4] is likely to have a lower measurement error than the WHO discriminating-dose bioassay, as each assay combines 3–6 repetitions across different insecticide intensities. A dataset was analysed that contained intensity bioassay results from the Presidents Malaria Initiative, WHO Malaria Threats database [45], and studies collated by Moyes et al. [38]. There were insufficient data available that directly compared different Type I and Type II pyrethroids in the same experiment. To overcome this, data were pooled at the country level to compare studies that tested permethrin and deltamethrin. This dataset consisted of 4745 individual mortality estimates from 1583 intensity bioassays across 18 countries in sub-Saharan Africa. Data from CDC bottle bioassays and WHO tube assays were analysed separately to investigate pyrethroid-specific resistance in wild mosquito populations. The analysis was restricted to the African continent due to the availability of data.

A Bayesian binomial model was developed to generate dose-response curves from raw intensity bioassay data. Separate curves were originally fit for each insecticide to the whole dataset to illustrate overall trends (Figure 10). Separate models were then fit to each set of concentrations to estimate the individual median lethal concentration (Lc_{50}; i.e., the concentration at which 50% of mosquitoes tested died). Mean LC_{50} estimates by country and year were calculated from individual estimates, with 95% credible intervals generated using bootstrapping methodology.

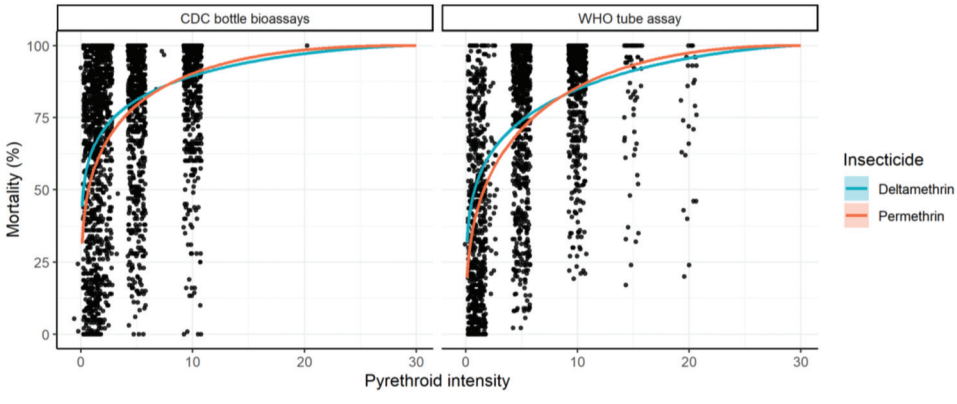

Figure 10. Overview of the intensity bioassay data used for analysis: points represent raw mortality data at each respective insecticide intensity with the modelled dose-response curve shown by Table 1 at 1×, 2×, 5×, 10×, 15×, or 20×.

Overall, across all data the dose-response curves were similar. On average, at an exposure of up to 10× the DD, the best-fit curve indicated that mortality induced by deltamethrin was higher than that of permethrin (Figure 10). This overall consistently shaped dose-response curve is compatible with the hypothesis that the two insecticides have different DDs. If the concentration of permethrin originally selected as "discriminating" induces a higher level of mortality than that selected for deltamethrin, then this discrepancy will be propagated across all concentrations (as they are relative to the DD at 2×, 5×, and 10×). The combined curves suggested that this is reversed when extrapolated to higher intensities, though this is likely an artefact of the shape of the curve used to

describe the dose–response relationship combined with relatively few data points above 10× concentrations, though this needs to be verified.

When analysing data at the country level, different trends were observed in different countries (Figure 11). High LC_{50} variability was seen in all locations, with substantially greater differences seen within-country than between countries. On average, differences between insecticides appeared marginal, with many overlapping mean LC_{50} estimates across both insecticides. Lower LC_{50} estimates (higher mortality) were seen for deltamethrin across most countries and assay types. There were several countries where this trend was, on average, reversed (i.e., higher LC_{50} estimates for deltamethrin in Burkina Faso and Cote d'Ivoire for WHO tube assays). Nevertheless, the difference between the LC_{50} estimates of the two insecticides was substantially less than the differences seen between assays of the same insecticide conducted in the same country, suggesting high variability but no clear pattern.

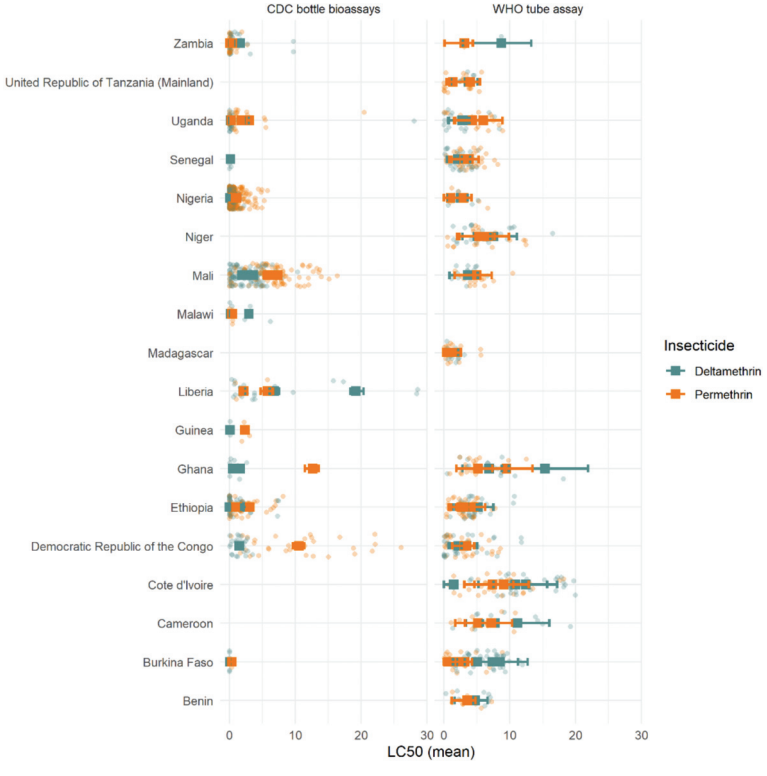

Figure 11. Estimated LC_{50} values for each country and assay type. The solid squares represent the mean value per country with each individual intensity bioassay LC_{50} value shown as the light points. Horizontal coloured lines indicate 95% credible intervals of the mean LC_{50} estimates. The x-axis units are DD concentration 10×, 20×, and 30×.

If there were differences in the suite of resistance mechanisms against Type I and Type II pyrethroids, and these mechanisms were established in populations, then it might be expected that resistance would diverge over time if selection pressures were continual. Selection is thought to be driven, at least in part, by ITN use, so this selection pressure is likely to be relatively consistent, as ITNs are typically replaced every three years. Differences in time were difficult to discern from these resistance-intensity data, as results were only available for 1–5 years. Nevertheless, the results were surprisingly consistent over the

different years, with those countries showing differences between pyrethroids generally persisting (Figure 12). On average, differences in mortality between insecticides did not increase over time, providing support for the cross-resistance hypothesis.

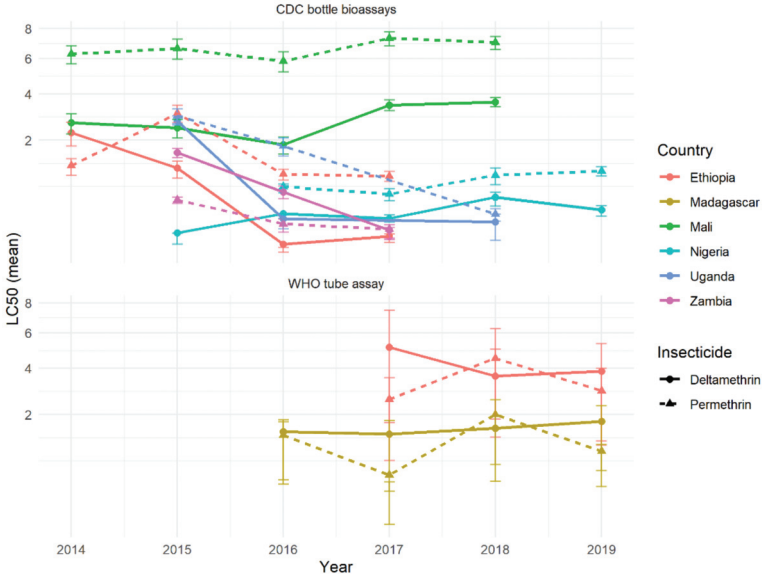

Figure 12. Country-specific mortality trends over time for countries with data from 3 or more consecutive years for both insecticides (per assay type). Mean LC_{50} values for each insecticide per country are shown for each assay and year. Different colours represent different countries, and each insecticide is shown by point and line type (permethrin in triangles and dashed lines, and deltamethrin in point and solid lines). Ninety-five credible interval estimates for the mean LC_{50} estimates are shown with the vertical whiskers, whilst horizontal lines link countries (though sites varied within countries over time).

Importantly, the difference between insecticides was likely not substantial enough to have a meaningful public health impact. The absolute difference in mortality at the DD dose predicted by the dose-response model was relatively low, varying from 2–27% between countries with multiple years of data (data not shown). Temporal trends, when they did appear, were also relatively minor, changing on average by only a small percentage over the timeframe. Evidence from the CDC bottle assay in Mali consistently showed higher mortality after deltamethrin rather than permethrin exposure, which remained constant over multiple years (Figure 11). However, a negligible difference was seen in the WHO tube assay from the same region (Figure 11), so it is unclear whether this may be due to a sampling/procedural artefact or differences within the country.

Whilst intensity bioassays may help in decreasing measurement errors compared to DD bioassays, the phenotypic field data remains very noisy. Whether these differences represent true variability in the local mosquito populations or are an artefact of the assay is unclear. Overall, these data indicated there was no consistent difference in mortality between deltamethrin and permethrin.

8. Do Mosquitoes, Resistant or Susceptible, Exhibit Different Behavioural Responses to Different Pyrethroids?

Vector populations can respond to IRS or ITN selection pressure with changes in behaviour, such as shifts in time or location of biting, resting site preferences, or host preference to avoid encountering the insecticide. However, behavioural resistance may

have many other less apparent forms; e.g., changes in sensitivity to repellent or irritant properties, or modified blood-feeding behaviours. Potentially, less detectable changes might be associated with highly visible secondary consequences; e.g., a thicker cuticle due to resistance could result in changes in flight behaviour. If there are differences in behavioural responses to different pyrethroids, then behavioural resistance might diverge such that resistance to one pyrethroid might be overcome by deploying a different one, or deployment choice might need to consider whether certain pyrethroids are more or less likely to drive behaviour in resistant mosquitoes that could lower an ITN's efficacy.

The mechanisms of insecticide resistance in malaria vectors are being studied and characterised extensively at the molecular level. Yet, knowledge of behavioural change associated with resistance is relatively poor, and few studies have directly compared the behavioural response to different pyrethroids within the same study. Before the emergence of resistance, an early hut trial in The Gambia concluded that permethrin was the most repellent pyrethroid, followed by λ-cyhalothrin, deltamethrin, and lastly cypermethrin [46]. Over a decade later, Hougard et al. [47] tested bednets with various combinations of bifenthrin and carbosulfan against both resistant and susceptible *An. gambiae* s.l., and reported no differences in entry rates between treatments or vector populations. Asidi et al. [48] tested bednets treated with α-cypermethrin, λ-cyhalothrin, permethrin, deltamethrin, or carbosulfan against resistant *An. gambiae* s.l. in Côte d'Ivoire. Here, all nets performed similarly, with none exhibiting any deterrent effects until they had been washed, after which all treatments reduced entry rates by approximately half. Cooperband and Allan [49] found *An. quadrimaculatus* spent significantly longer times resting on surfaces treated with deltamethrin than with bifenthrin or λ-cyhalothrin, but only after initial contact was made. Hughes, Foster, et al. [50] found no evidence for deterrence in *An. gambiae* s.l, but recorded lag times between first net contact and the start of blood feeding of 1 min with untreated nets, and 2.5 and 3 min for Olyset and PermaNet 2.0 nets, respectively. Other studies have described the behavioural responses of mosquitoes to pyrethroid-treated nets. However, there was great variability in the study designs, behavioural definitions, net treatments, and mosquito species reported in the literature. Studies investigated numerous diverse wild vector populations at different locations, on different dates, with very different or uncharacterised levels of resistance. Consequently, the results were highly variable, with little indication of a conclusive trend among the behavioural responses elicited by individual pyrethroids, let alone anything to distinguish behaviours unique to different insecticides within the pyrethroid class.

9. How Suitable Are Existing Resistance-Monitoring Methods for the Detection or Measurement of Behavioural Resistance?

Since the impact of any insecticide-based control method is determined ultimately by the mosquitoes' behavioural response at or near the interface of insecticide delivery, the selection of ITNs should ideally be based on evidence derived from appropriate assays that capture the range of behaviours that influence the ITN's performance. The discriminating-dose and resistance-intensity bioassays (whether WHO tube or CDC bottle) currently used to monitor resistance were not designed to allow for or monitor behavioural variation.

Bioassays such as the cone and tunnel tests record knockdown or mortality (and blood-feeding rate in tunnel tests) of young adult female mosquitoes following unnaturally high levels of exposure to an active ingredient under highly artificial conditions; i.e., forced, without the presence of a host, or using a non-natural animal host. Measuring the efficacy of insecticides in such an environment will not predict how the eventual insecticidal net products or residual spray preparations will perform under field conditions, hampering informed deployment decisions. Similarly, a change in behaviour in a mosquito exposed to a pyrethroid might well confound the results of such bioassays. For example, an increase in sensitivity could reduce contact times at the treated surface, resulting in lower mortality, whereas an increase in tolerance could increase the contact time and mask existing resistance.

Discriminating-dose assays (WHO tube and CDC bottle) were intended as a litmus test for the emergence of resistance in mosquito populations to evaluate fast-acting pyrethroids before the extent of resistance seen in Africa today became established. They were not designed to measure quantitative differences between mortality rates to inform product choices, and certainly not to draw any comparisons between the efficacy of different products. Their limitation is their inability to capture the full range of possible behavioural and sublethal effects—such as impacts on longevity, reproductive output, or development of *Plasmodium* spp. Improving, augmenting, or replacing tests as affordable, rapid, and simple as the existing WHO tests with new assays that retain those properties, as well as adding the ability to capture, distinguish, and measure a range of outcomes without ambiguity, will be a challenge.

Carrasco et al. [51] attempted to capture all anticipated behavioural events and other potential outcomes following insecticide vector control within a framework to guide classification and investigation. A better understanding of behavioural responses, to both insecticides and specific products, and how they differ between susceptible and resistant mosquitoes should inform the deployment of the most effective products. Ideally, given the vast range of behaviours that could be impacted and might need to be quantified, resistance-monitoring efforts should focus methods to detect changes in those behaviours most likely to affect a product's performance. To meet this need, there have been a number of advances in development of novel methodologies [50,52–54] to collect the essential data about the behavioural responses of *Anopheles* under more operationally relevant conditions, including large-scale testing arenas [55,56].

10. Discussion

10.1. The Evidence for Divergent Resistance within the Pyrethroid Class

Molecular analysis of metabolic resistance, together with analysis of phenotypic resistance in mosquito populations (including analyses of intensity data, of diagnostic dose bioassay data from populations that have been tested with multiple pyrethroids, and of spatiotemporal trends), provide evidence that there is strong cross-resistance among pyrethroids, particularly between permethrin and deltamethrin. P450s SAR (structure–activity relationship) findings concluded that the more commonly used pyrethroids examined were the most vulnerable to metabolic attack (by cytochrome P450s), while bifenthrin, λ-cyhalothrin, and α-cypermethrin were less vulnerable to metabolic attack. Bioassay data from *Aedes aegypti* and *An. sinensis* suggested that bifenthrin may demonstrate relatively low cross-resistance with other more commonly used pyrethroids. Bifenthrin has not been widely used in malaria control in Africa and no discriminating dose has been defined, but its potential use in malaria vector control warrants further investigation. There is also evidence that resistance to etofenprox could diverge from resistance to the more commonly used pyrethroids; however, further investigation regarding vulnerability to metabolic attack by P450s is required.

In field populations, variability in discriminating-dose and dose-response assay mortality was high. This variability was predominantly at a fine geographical scale (i.e., assays done within 50 km of each other were highly variable), indicating that if there were a difference between Type I and II pyrethroids, it would be very local and beneath the size of the regions to which insecticidal nets are currently allocated. There was good evidence that the mortalities from exposure to deltamethrin, permethrin, α-cypermethrin, and λ-cyhalothrin were strongly correlated across *An. gambiae* s.l. populations. These correlations were also seen for deltamethrin, permethrin, and λ-cyhalothrin (α-cypermethrin was not tested) in the *An. funestus* subgroup, and in all three of the main malaria vectors within the *An. gambiae* complex.

10.2. The Suitability of Current Testing Methods to Monitor Insecticide Resistance and Make Vector-Control Decisions

Deployment decisions for ITNs are being guided by information arising from the discriminating-dose and resistance-intensity bioassays, but it is not clear how well differential mortality in WHO tube or CDC bottle bioassays predict how well an ITN treated with one or another pyrethroid will perform in a specific site. Bioavailability may play an important role in the relative efficacy of different ITNs, and testing for the relative performance of different nets against field populations would provide more directly relevant information for deployment decisions, alongside or in place of conventional bioassays. Given the limited products available for vector control, as well as narrow collection of available chemistries, programmes must make ITN deployment decisions based on the data that can realistically be collected. The current monitoring system for insecticide resistance is imperfect and should be adapted to make better use of the available resources, while being mindful that limited mosquito collections preclude the testing of all insecticides/products.

In general, following exposure of characterised lab strains in WHO tube bioassays under controlled conditions, intrastrain mortality to permethrin, deltamethrin, and α-cypermethrin were similar. However, in intermediately resistant strains, some divergence in mortality rates was observed. However, importantly, the level of variability in observations of mortality between tubes (measured using standard deviations) was also greater in these intermediately resistant strains, which reduced certainty in apparent contrasts between insecticides. Discriminating-dose assays are poor tools for quantitative analysis of resistance levels where resistance is established, producing the most variability in results in laboratory colonies and field populations where resistance was moderate and mortality was intermediate, which is likely to be the case for all or most pyrethroids in most populations of African malaria vectors. For comparisons across insecticides, intensity assays (e.g., 1×, 5×, 10×) suffered from the same problem as the discriminating doses on which they depended—an apparent lack of parity across pyrethroid insecticides. Quantitative dose-response assays, which do not depend on a discriminating doses, are recommended for robust comparisons between insecticides.

All bioassays are vulnerable to ambient conditions, including humidity and temperature, in addition to other environmental effects more easily standardized by the user. It is crucial that deviations from optimal conditions are reported, along with the improved provision of sampling details, to understand extrinsic factors that could influence bioassay results.

11. Conclusions

Evidence suggests that in areas where pyrethroid resistance exists, different results in insecticide susceptibility assays with specific pyrethroids currently in common use (deltamethrin, permethrin, α-cypermethrin, and λ-cyhalothrin) are not necessarily indicative of an operationally relevant difference in potential performance. Consequently, it is not advisable to use rotation between these pyrethroids as an insecticide resistance management strategy. Less commonly used pyrethroids (bifenthrin and etofenprox) may have sufficiently different modes of action, though further work would be needed to examine how this may apply to insecticide resistance management.

Supplementary Materials: The following are available online at https://www.mdpi.com/2075-4450/12/9/826/s1, Figure S1: Dose response curves for deltamethrin (A–M) and permethrin (N–Y) using original raw data from the 1998 WHO multicentre study [8]; Table S1: Probit analysis of deltamethrin and permethrin using original raw data from the 1998 WHO multicentre study [8]. Analysis was conducted using PoloJR program within PoloSuite (Ver 2.1). The discriminating dose is twice the LD99. Abbreviations: LD = Lethal dose, DD = Discriminating dose; Figure S2: Calculated discriminating doses (%) for (A) deltamethrin and (B) permethrin. Points show individual sites/strain combinations, and data pooled by species or overall, by insecticide. Site/strain testing <3 concentrations of an insecticide and datasets not robust enough to calculate lethal dose matrixes are excluded. Discriminating doses are set at 2 x the calculated lethal dose at which 99% (LD99)

of test mosquitoes were killed. LSHTM *An. stephensi* Beech data included; Figure S3: Mosquito mortality (%) following exposure to deltamethrin in WHO tube bioassays of site/strain combinations for (A) *An. albimanus*, (B) *An. gambiae*, and (C) *An. stephensi*. Numbers above bars show number of exposed mosquitoes; Figure S4: Mosquito mortality (%) following exposure to permethrin in WHO tube bioassays of site/strain combinations for (A) *An. albimanus*, (B) *An. gambiae*, and (C) *An. stephensi*. Numbers above bars show number of exposed mosquitoes. Table S2: Summary statistics of variability in mosquito mortality following exposure to pyrethroids in standard WHO tube or CDC bottle bioassays. Mosquitoes were exposed to insecticides following the recommended methods for each test and mortality in each replicate tube or bottle was recorded 24-h post-exposure. The strains detailed here are those maintained by the Ranson Group or LITE at LSTM, UK. Abbreviations: R = Insecticide resistant mosquito strain, S = Insecticide susceptible mosquito strain, IKR = inter-quartile range. Table S3: *p*-values (Welch's *t*-test) comparing mean mosquito mortality following exposure to α-cypermethrin 0.05%, deltamethrin 0.05%, or permethrin 0.75% in a standard WHO tube bioassay. Values significant at the 5% level ($p = < 0.05$) are highlighted in bold. Abbreviations; Delta = deltamethrin, Perm = permethrin, Alpha = α-cypermethrin; Figure S5: Mosquito mortality over time following exposure to pyrethroids in a standard WHO tube bioassay. Ranson group (A–J) and LITE (K–O) mosquito strains were exposed to deltamethrin 0.05%, permethrin 0.75% and α-cypermethrin 0.05% in a standard 1-h WHO tube bioassay, and their 24-h mortality was recorded. Coloured circles indicate each individual replicate tube; Figure S6: Box plot summarising mosquito mortality following exposure to deltamethrin 0.05% (top), permethrin 0.75% (middle), or α-cypermethrin 0.05% (bottom) in a standard WHO tube bioassay in Ranson group strains. Each box represents a different mosquito strain. Coloured circles and n-numbers indicate each individual tube replicate; Figure S7: Box plot summarising mosquito mortality following exposure to deltamethrin 0.05% (top), permethrin 0.75% (middle), or α-cypermethrin 0.05% (bottom) in a standard WHO tube bioassay in LITE strains. Each box represents a different mosquito strain. Coloured circles and n-numbers indicate each individual tube replicate; Figure S8: Distribution of larval relatedness of (A) An. gambiae and (B) An. Coluzzii within breeding sites sampled across multiple locations in 2016 from southern Ghana. Individuals (median N = 7 per site) were genotyped by reduced coverage whole genome sequencing and pairwise relatedness categories estimated from chromosome 3 data (2229 SNP markers) as siblings or unrelated using ML-Relate [41]. The percentage of sibling relationships is shown. Sample site numbers are nominal and are not equivalent between plots.

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Mosquito-Textile Physics: A Mathematical Roadmap to Insecticide-Free, Bite-Proof Clothing for Everyday Life

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Simple Summary: Mosquitoes can bite across clothing and transmit disease. This is prevented with pesticides applied to clothing. We developed non-insecticidal cloth and garments that provided 100% protection, were comfortable and look-like and feel-like regular clothing.

Abstract: Garments treated with chemical insecticides are commonly used to prevent mosquito bites. Resistance to insecticides, however, is threatening the efficacy of this technology, and people are increasingly concerned about the potential health impacts of wearing insecticide-treated clothing. Here, we report a mathematical model for fabric barriers that resist bites from *Aedes aegypti* mosquitoes based on textile physical structure and no insecticides. The model was derived from mosquito morphometrics and analysis of mosquito biting behavior. Woven filter fabrics, precision polypropylene plates, and knitted fabrics were used for model validation. Then, based on the model predictions, prototype knitted textiles and garments were developed that prevented mosquito biting, and comfort testing showed the garments to possess superior thermophysiological properties. Our fabrics provided a three-times greater bite resistance than the insecticide-treated cloth. Our predictive model can be used to develop additional textiles in the future for garments that are highly bite resistant to mosquitoes.

Keywords: mosquito; bite-proof garment; model; textile; non-insecticidal; physical barrier

1. Introduction

Mosquito-transmitted diseases are a major, global human health problem [1]. Pathogens transmitted by mosquito bites cause illnesses that kill an estimated 700,000 people each year [2]. Personal protection from mosquito-borne diseases has largely involved the use of chemical repellents applied to clothing and skin or insecticides either sprayed on garments before use or bound to textiles or garments to survive multiple uses and washes. Insecticide-treated textiles in the form of long-lasting insecticidal bed nets (LLINs) are also used for mosquito control in malaria-endemic areas. According to the World Health Organization, pyrethroid-treated bed nets have played a vital role in reducing malaria in Africa

(World Health Organization (2019), World Malaria Report, WHO, Geneva, Switzerland [3]). Between 2000 and 2015, an estimated 663 million clinical cases of malaria were averted, of which 68% were attributed to the wide-area deployment of LLINs [4]. The use of insecticide-treated curtains [5,6], long-lasting insecticidal bed nets, and insecticide-treated clothing [7] have substantially reduced the transmission of vector-borne pathogens. Unfortunately, the widespread use of insecticides has also led to the development of insecticide-resistant mosquitoes, and the insecticides are now ineffective in many places [8].

Furthermore, in spite of the benefits from insecticide-treated textiles, there are potential deleterious health effects [7]. Since the garments are in continuous contact with the skin, the potential for insecticide exposure is increased. Permethrin is the principal insecticide used to treat clothing [9]. Development of safe, alternative insecticides for textiles is costly and requires regulatory approvals for new chemistry. Because of the potential health risks from the use of pesticides, people today given a choice prefer to avoid insecticide exposure. Development of mosquito-bite-resistant garments without insecticides that are comfortable and as effective (or more effective) than insecticide-treated garments would be a "game changer" and provide to the public, for the first time, a choice. We have achieved this objective.

Fabrics inherently are favorable structures for producing physical barriers against insects. Textiles have a three-dimensional structure assembled with interlacements or intermeshing fibers and yarns in organized patterns [10]. The design of fibers and yarns produce textile structures with a diverse range of properties, some of which could provide insect protection [11]. Fabrics have been specifically designed as physical barriers against environmental factors such as water [12], airflow [13], or heat and cold [14]. The existence of open spaces between fibers and yarns ensures fabric breathability and thermal comfort [15]; however, these spaces produce pores through a fabric allowing penetration of human olfactory (smell) and thermal (temperature) cues that attract mosquitoes [16]. The fabric pores serve as channels for the mosquito to take a blood meal. The objective of our research is to develop a mathematical model to predict blood feeding across textiles that could be used to develop a practical, non-insecticidal, bite-resistant garment.

2. Materials and Methods

2.1. Mosquitoes

Adult, female yellow fever mosquitoes, *Aedes aegypti* (Diptera: Culicidae), are a major vector of pathogens that cause animal and human diseases worldwide [17–19] and were used as a model insect for the studies that follow. *Ae. aegypti* females (Figure 1A and Figure S1) were obtained from a colony maintained in the Dearstyne Entomology Laboratory at North Carolina State University, Raleigh, NC, USA. The mosquito colony has been continuously reared for approximately 5 years and is free of pathogens. Adults were kept at 27 °C and 80% relative humidity with a 14:10 h light: dark photoperiod. Adults were provisioned with a 10% sucrose solution (in distilled water) *ad libitum*. To obtain eggs for colony maintenance, female mosquitoes were fed porcine blood (obtained from a local abattoir) using an *in vitro* blood-feeding device (described later). Larvae were kept under the same environmental conditions as adults and fed a porcine liver powder: brewer's yeast mixture (2:1, wt:wt). Larval rearing water was dechlorinated using a standard aquarium dechlorinating agent.

2.2. In Vitro Feeding/Bioassay System

An *in vitro* bioassay system was developed (shown in Figure S2A) to blood feed mosquitoes for routine colony maintenance and to bioassay the barrier materials for bite resistance. The major components of the system are a blood-feeding reservoir, Plexiglas® cage, and a circulating water bath for regulating the temperature of the blood. The blood-feeding reservoir is designed to contain the blood, fix a feeding membrane over the blood, and fix barrier materials on top of the feeding membrane for bioassays [20]. Briefly, the blood reservoir (16.5 cm length × 3.5 cm width × 0.5 cm depth) was produced with a hand-

held router from a rectangular block of Plexiglas® (28 cm length × 5.5 cm width × 1 cm thickness). A hole (4 mm diameter) was drilled at the center of the top and bottom edge through the plastic into the blood reservoir. A tap was used to cut threads into the plastic so that a valve could be screwed into the top and bottom holes. Two holes (each 4 mm diameter) were drilled from the bottom edge of the device through the plastic to the blood reservoir. A loop of stainless-steel tubing (3 mm diameter) was placed into the blood reservoir, and the tubing was inserted through the holes so that the cut ends protruded out of the plastic. Epoxy cement was used to seal the tubing in place inside the blood reservoir of the device. The ends of the tubing were connected to a circulating water bath to heat the blood.

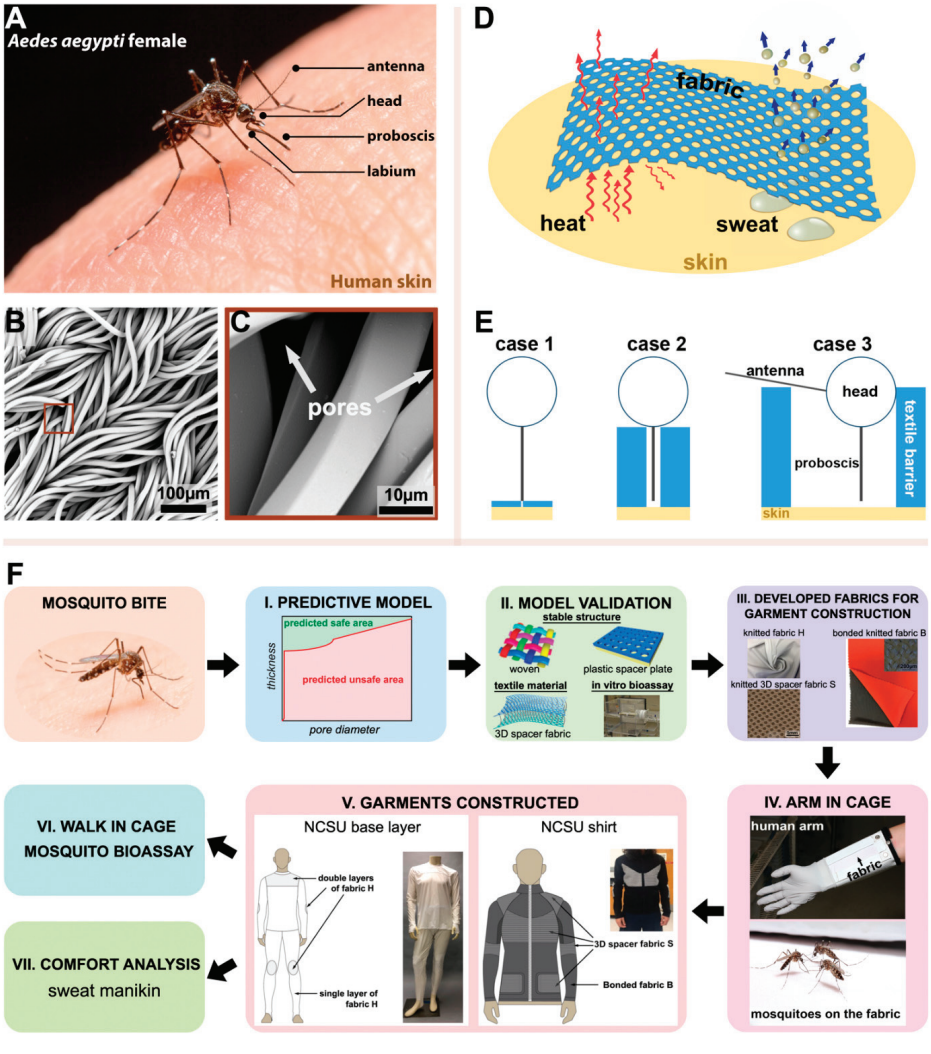

Figure 1. Principle of a bite-resistant textile structure against *Aedes aegypti*. (**A**) An *Ae. aegypti* adult female feeding on the blood beneath human skin. (**B**) SEM image of a knit structure. (**C**) Example of pores formed by the filaments in the knit structure. (**D**) Heat and moisture management of a fabric. (**E**) The proposed three cases for mosquito-bite resistance. (**F**) Research steps for the design of bite-resistant garments.

For blood feeding, a transparent collagen film (product code 894010.95; Devro, Inc., Columbia, SC, USA) was hydrated in distilled water and stretched over the top of the device. A gasket, cut from a sheet of cork-rubber composite (Fel-Pro, part no. 3019; AutoZone, Raleigh, NC, USA) was placed on top of the collagen film. A rectangular piece of plastic (3 mm thick) the size of the blood-feeding device was then placed on top of the gasket. The central area of both the rubber gasket and plastic frame was removed so that the collagen film is fully exposed to the mosquitoes. Metal binder clips hold the gasket and frame in place on top of the blood-feeding device, preventing leakage of blood. A 30 mL syringe filled with blood was then attached to the valve that was screwed into the top hole of the blood-feeding device. With the device tilted at a slight downward angle, the blood was slowly transferred into the reservoir. The valve attached to the bottom of the device was opened to allow air displacement as the blood is added. When the device was filled with blood, both valves were closed, and the circulating water bath was started to warm the blood to 35 °C.

The barrier materials (for example, the plastic blocks shown in Figure S2C; the barrier materials tested are in toto listed in Table 1) to be evaluated for bite resistance were cut exactly to fit over the collagen film within the plastic frame. The test area for the in vitro bioassay was the same as that for the arm-in-cage studies discussed later. Masking tape, placed around the inner edges of the plastic frame, slightly overlaps the barrier. In this way, mosquitoes are prevented from gaining access to the collagen film by probing around the edges of the barrier. The blood-feeding device was inserted into a Plexiglas® bioassay cage (30 cm square on each side; Figure S2A) which contains mosquitoes for feeding (with the barrier material absent) or bioassay (when the barrier material is in place). For routine colony maintenance, the feeding membrane was not covered with barrier materials.

Prior to testing the barrier materials and inserting the blood-feeding device into the cage, 100 *Ae. aegypti* females were transferred to the bioassay cage (Plexiglas®, 30 cm on each side). Mosquitoes were starved overnight (sugar water removed from their rearing cage; females not blood fed) prior to testing. Female mosquitoes were 6–7 days of age (post emergence). Porcine blood obtained from a local abattoir was used in our bioassays. At the time of blood collection, sodium citrate was added as an anticoagulant. Just prior to initiating the bioassay, ATP (Sigma) was added to the blood (2.5 mg/mL) as a phagostimulant [20]. Each bioassay was conducted for 10 min., during which the number of times females landed and probed the barrier material was counted. A single event was recorded if a female landed and then inserted or attempted to insert her proboscis into the barrier material, regardless of whether the female probed multiple times after landing. A video recording was made of each bioassay so that the mosquitoes' responses to the surface of each barrier and probing behavior could be studied. At the end of the exposure period, mosquitoes were removed and killed in a freezer. Subsequently, each mosquito was crushed on a sheet of white paper to determine if she was able to probe through the barrier and obtain a blood meal. Blood spots on the paper were counted, and the percentage of mosquitoes that were blood fed was calculated based on the total number of mosquitoes released into the cage. The *in vitro* bioassays were repeated for each barrier material a minimum of 3 times. For routine blood feeding for colony maintenance, the number of mosquitoes in the cage was variable (50 to 200), and the feeding time extended until all of the mosquitoes that want to feed have time to feed to repletion. All bioassays and mosquito adult feeding, including the *in vitro* and *in vivo* (described later) tests, were conducted in the mosquito insectary laboratory at the Dearstyne Entomology Building of NC State University, at a temperature of 27–29 °C and 75–80% humidity. All tests were conducted during the photophase under florescent lighting.

2.3. In Vivo Bioassay for Bite Resistance

Measurement of the *in vitro* mosquito-bite resistance of the barrier materials was standardized in terms of the apparatus architecture (dimensions and exposed area of the feeding membrane) and blood-feeding conditions. Similarly, for the *in vivo* studies, the

dimensions of the bioassay cage and cloth area exposed for mosquito probing were the same. Our IRB for the *in vivo*, arm-in-cage studies required us to demonstrate *in vitro* bite resistance of greater than 80% for the barrier materials before conducting an *in vivo* test on the same barrier material. This restriction was to limit the potential number of mosquito bites received by the human subject. *In vivo* tests using human subjects is a more rigorous test of a fabric's bite resistance because of the volatile attractants emitted from the skin. *In vivo* testing is critical to understanding whether a textile will prevent mosquito bites. Therefore, validation of our predictive model and development of textiles for garment construction (discussed later) required *in vivo*, arm-in-cage studies.

Table 1. Barrier materials studied, their abbreviation, measured thickness and pore diameter, model prediction, and bite-resistance bioassay results.

Group	Type	Name	Abbreviation	Thickness (mm), Pore Diameter (μm)	Model Prediction †	Bioassay Result ††
Materials for model validation (test, in vitro)	Stable structures	Case 1 woven filtration fabrics	W1	0.052, 25	unsafe	fail
			W2	0.040, 18	unsafe	fail
			W3	0.058, 16	safe	pass
			W4	0.082, 8	safe	pass
		Case 2 plastic plates	S1	2.1, 500	unsafe	fail
			S2	2.1, 800	unsafe	pass
			S3	2.5, 500	safe	pass
			S4	2.5, 800	unsafe	fail
			S5	2.5, 1250	unsafe	fail
		Case 3 plastic plates	S6	2.72, 800	safe	pass
			S7	2.75, 1250	safe	fail
			S8	3, 1250	safe	pass
	Textile materials	Case 1 fabrics	T1	0.29, 36	unsafe	fail
			T2	0.26, 16	safe	pass
		Case 2 spacer fabrics	T3	2, 120	unsafe	fail
			T4	3.2, 420	safe	pass
		Case 3 spacer fabrics	T5	2, 940	unsafe	fail
			T6	3, 770	safe	pass
Fabrics used in garments (test, in vivo)	Textile materials	Case 1 fabric H	H	0.3, 28	safe	pass
		Case 1 fabric B	B	0.68, 0	safe	pass
		Case 2 spacer fabric S	S	2.48, 420	safe	pass
Permethrin-treated fabric (test, in vivo)	Chemical-treated textile materials	InsectShield® T-shirt fabric	P	0.61, 90	unsafe	fail
Garments (test, walk-in cage)	Garments	Under Armour® men's base 1.0 crew	I	–	–	–
		NCSU base layer	II	–	–	pass
		Winter army combat shirt	III	–	–	–
		NCSU shirt	IV	–	–	pass

Note: † Model prediction means the bite resistance of each fabric predicted by the bite-resistance model. "Safe" means the fabric has 100% bite protection and "unsafe" means the fabric is predicted to allow mosquito biting (based on our bite-resistance model). †† Bioassay result is an actual measurement of bite resistance. For in vitro and in vivo tests, "Pass" means the fabric demonstrated at least 95% bite protection. For the walk-in-cage test, pass means no bites.

Arm-in-cage studies (apparatus used shown in Figure S3A) were conducted with informed consent using a protocol for use of human subjects in research approved by the NC State University Institutional Review Board (IRB #2925) [21]. The assay methodology was designed to mimic a textile worn on the forearm with the fabric in close contact with the skin. Odorants and heat from the skin can diffuse through the fabric attracting mosquitoes seeking a blood meal.

The sleeve device (Figure S3A), constructed from bioassay textiles, exposed the cloth surface through an opening that was identical in size as was used in the *in vitro* assays. The sleeve was constructed from a polyvinyl-coated roofing membrane, Samafil® (Sika Corp., Canton, MA, USA). The sleeve was cut into a trapezoidal shape to fit a human arm and

with a 16.5 cm × 3.5 cm opening in the center that corresponds to the size and shape of the opening in the *in vitro* blood-feeding device described earlier. A plastic frame was riveted to the sleeve to keep the exposure area of the textile from deforming when the sleeve was attached to the forearm of the study participant.

In total, 100 unfed, nectar-starved *Ae. aegypti* adult females were transferred to a bioassay cage 10–30 min before being assayed, as described earlier for the *in vitro* assay. The textile to be assayed was laid over the underside of the forearm of the study participant. The sleeve was laid on top of the cloth and attached to the participant's forearm with Velcro® straps. The hand of the participant was then covered with a nitrile glove to prevent mosquito bites on the hand. The bioassay was started when the participant inserted his/her arm through a cloth sleeve into the bioassay cage. An observer counted the numbers of mosquitoes landing on the cloth and probing during a 10 min exposure period, and in some cases video recordings were made of the inserted arm only as needed for further documentation. After the bioassay was terminated, mosquitoes were examined for blood feeding by crushing them on white paper as previously described for the *in vitro* assay. Blood spots on the paper were counted, and the percentage of mosquitoes that were blood fed was calculated based on the total number of mosquitoes released into the cage. The mosquitoes used, mosquito conditioning, the number of mosquitoes, and level of replication were the same as that described for the *in vitro* assay.

2.4. Walk-in-Cage Studies of Whole Garments

A garment is composed of integrated fabrics and seams that have various rectilinear and curvilinear pattern pieces needed to conform to differing human body shapes. The gap distance between the garment and the skin varies throughout the body and can change with posture along with textile stretching, all of which can affect bite resistance. These factors affect the fabric performance regarding mechanical bite resistance and comfort, which can only be evaluated through whole-garment testing. Walk-in-cage studies provide a method for testing garments under quasi-field conditions with higher mosquito-bite pressures. We also avoided disease risks to human subjects that might occur using wild mosquito populations in a field test.

Garments (Figure S6A,B, described later in detail, and all the garments tested are listed in Table 1) were tested in a walk-in enclosure (2 m height × 4 m length × 4 m width) constructed from polypropylene screens (mesh size 1.8 mm; Lumite Company, Alto, GA, USA) that were sewn together to form a cage. The test cage had a zippered opening and was supported with a 2 inch × 4 inch wooden frame. The bottom edges of the panels were taped to the cement floor to prevent mosquitoes from escaping. The cage was covered with white bed sheets and then an outer layer of black plastic to block external light. Light inside the cage was provided by a single 35 W fluorescent tube placed at each corner suspended from the ceiling. Prototype garments were worn by a human subject with informed consent with an approved research protocol (IRB# 9075) from the NC State University Institutional Review Board. For the prototype base layer garment, the subject's head and neck were protected by a bee veil, the hands were covered by nitrile gloves and the feet covered with shoes. Each pant's leg was taped to the shoe to prevent biting at the margin between the pants and shoe. For the prototype NCSU shirt, the subject wore three pairs of pants that combined were 100% bite proof; otherwise everything was the same as for the base layer.

At the beginning of the trial in the bioassay cage, 200, 6–7-day-old, unfed adult female *Ae. aegypti* were released by the test subject. The condition of the mosquitoes was described earlier. In the bioassay cage, the subject stood motionless with arms at her/his sides for 10 min and then sat with arms crossed for an additional 10 min on a waist-high stool (no back support). In a sitting position, the fabric was stretched at the knees, elbows, and shoulders. These two postures mimicked how a garment would be worn for mosquito protection. The postures caused the garment to deform, changing the gap distance between the fabric and skin on different parts of the body, thus potentially affecting bite-resistance performance. Assays were conducted during the photophase at 25–28 °C and a relative

humidity of approximately 30–40%. At the end of each trial, the subject exited the bioassay cage, and all mosquitoes were collected with a mechanical aspirator and killed in a freezer. After removing the garment, the test subject's skin was examined for mosquito bites with the assistance of another researcher. Areas of the body where bites occurred were recorded so that the corresponding areas of the garment could be reinforced to prevent bites in subsequent prototypes. Mosquitoes were collected, frozen, and examined for blood feeding by crushing them on white paper, as described earlier. Each garment was evaluated in a minimum of three separate trials conducted on different days.

2.5. Model Rationale and Mosquito Morphometrics

Blood feeding of mosquitoes on humans involves physical interactions between the mosquito's external morphology associated with the head and exposed skin, requiring a combination of insect behaviors allowing the mouthparts to penetrate the cornified, squamous epithelium and insert into the host blood vessels near the skin surface. When a textile is placed over the skin, the fabric restricts access to the skin and affects mosquito landing and probing behaviors. This creates another compliment of physical interactions between the textile and the mosquito that affects differently how the mosquito also interacts with the skin below. These physical parameters of the mosquito's head and mouth parts impose three-dimensional limits, defined by their shape and size, on a mosquito's ability to penetrate the textile and the skin. Understanding these limits and the mechanics of biting affected by the physical structure of cloth and the morphometrics of the mosquito's feeding structures can be used to develop textiles to optimally resist blood feeding, as well as providing optimal comfort without the need for insecticides or repellents.

The mosquito proboscis (Figure S1A,B) is a collection of interlocking needle-like mouthparts (stylet in shape) covered by a sheath, the labium. The stylets consist of the labrum (Figure S1C,D), a pair of mandibles, a pair of maxillae, and a hypopharynx extending from the floor of the mouth between the mandibles and maxillae. The rigid, pointed labrum tip is shown in Figure S1D and is the first part of the proboscis that makes contact with skin to initiate biting. The other mouth parts are used to advance the insertion into the skin and for channeling blood to the mouth. Preventing labrum penetration and/or contact with the skin prevents blood feeding.

Our model to describe the physical interactions between a mosquito and a barrier material is divided into three Cases that represent the process of fabric penetration to obtain a blood meal and how the mosquito interacts with different textile surfaces. For our Case 1 model (Figure 1E), the dimension of the labrum (the largest mouthpart needed for penetration of the skin and blood feeding) is a critical attribute of the mosquito's mouthparts. To measure its dimensions, the labrum from 20 adult female mosquitoes (described before) was dissected using needle-point forceps, then gold coated using a SC7620 Mini Sputter Coater (Quantum Design GmbH, Darmstadt, Germany), visualized using a Phenom G1 desktop scanning electron microscope (SEM; Thermo Fisher Scientific Inc., Waltham, MA, USA) in the Phenom SEM and Forensic Textile Microscopy Laboratory at NC State University, and the measurements of maximum labrum diameter (D), labrum tip angle (α), and tip length (L_{tip}) taken from these images. To avoid body shrinkage from dehydration, the mosquitoes were killed by freezing, and the mouth parts were quickly dissected and gold coated.

For the model for Case 2 and Case 3 (Figure 1E), 20 adult females were used for measurements of the head diameter (D_{head}) and antenna length (L_{antenna}), not including the flagella branches and proboscis length ($L_{\text{proboscis}}$), using a Nikon SMZ-1000 Zoom Stereo Microscope fitted with an ocular micrometer (Nikon Metrology, Inc., Brighton, MI, USA) in the Phenom SEM and Forensic Textile Microscopy Laboratory at NC State University. To avoid body shrinkage from dehydration, the mosquitoes were killed by freezing and then morphometric measurements were immediately taken. The mosquito anatomy that was measured is shown in (Figure S1B,C).

2.6. Model Development

Based on observations of mosquito probing and biting behavior, we hypothesized that the morphometrics critical for blood feeding were associated with the head size and length, the relationship of the antennae to the head, and the length and diameter of the labrum. Based on these assumptions, there were three rationales on how a textile might be used to prevent penetration of the skin: (i) a barrier that is thick enough to prevent the labrum from reaching and penetrating the skin; (ii) a barrier with small enough pores that prevented the labrum and/or the head from penetrating the surface of the textile; and (iii) combinations of (i) and (ii). The boundaries for thickness based on our morphometrics were set from 0 to 2.95 mm (the sum of the head diameter and proboscis length) and the boundaries for pore diameter were from 0 μm to 1.8 mm (the sum of the antenna length and head diameter). Due to the complex geometry between the head and proboscis, we specified three cases to achieve a bite-resistant structure: pore diameter smaller than the diameter of the labrum, pore diameter smaller than the head diameter, and pore diameter smaller than the sum of the head diameter and antenna length. In those cases, each pore diameter has a specific thickness determined by the geometry of the mosquito mouthparts, head, and antenna that would impact biting.

The bite-resistance model describing the relationship between the pore diameter and thickness of a textile barrier is shown in Figure 2B–D. In Case 1, the critical trajectory of the combination of pore diameter and thickness is the hypotenuse of a right-angled triangle (the longest side) of the labrum. In Case 2, the critical factor is the arc determined by the head shape. In Case 3, the critical factor is a straight line governed by the antenna. Based on this geometry, we defined the mathematical relationships for each case.

2.7. Materials for Model Validation

2.7.1. Stable Structures

Due to the sophisticated interlacement and entanglement of the fibers [22], most textiles have irregularly distributed pores of different shapes and area and an uneven thickness. In terms of the latter, a textile never has an absolute planer surface. Because of this variability, relating textile structure to bite resistance is not precise. This is further complicated by the large variety of possible textile structural parameters that can be selected, including yarn denier, covering rate, surface roughness, weave or knitting density, etc. Therefore, the use of a textile with a single pore shape and size and a single, fixed thickness is challenging and requires testing a vast number of iterations using different textile production methods. Instead, our first step in model validation was the use of stable structures.

For Case 2 and Case 3 conditions, we simulated a porous fabric with rigid polypropylene plates (Figure S2C) with bored holes of varying diameters that were distributed in uniform patterns on each plate where we could simulate precise pore shapes (circular), pore areas, and textile thicknesses. The size of each polypropylene plate was fixed at 14.5 cm × 3.4 cm to fit into the *in vitro* bioassay device described earlier. Based on mosquito morphometrics, we focused on 3 different pore diameters which (i) included the head (1.25 mm); (ii) partially excluded the head (0.8 mm); and (iii) completely excluded the head (0.5 mm). Those plates were produced by a combination of 3D printing to obtain the correct thickness and computer numerical controlled (CNC) machining to obtain a specific pore size and number of holes. First, a plain mold was printed on a 3D printer (Objet Connex350, Edward P. Fitts Department of Industrial and Systems Engineering, NC State University, Raleigh, NC, USA) to the desired thickness. Then the pre-designed pattern was processed on a CNC machine to obtain holes with precise diameters that would mimic a porous textile. A series of prototype spacers (S = plastic spacer; S1, S2 . . . , S8, listed in Table 1) were made at different combinations of pore sizes and thickness, which spans Case 2 and Case 3's safe and unsafe combinations. As shown in Figure S5C,D, S1 is 2.1 mm thick, with a 0.5 mm pore diameter; S2 2.1 mm thick, with a 0.8 mm diameter; S3 2.5 mm thick, with a 0.5 mm diameter; S4 2.5 mm thick, with a 0.8 mm diameter; S5 2.5 mm thick, with a

1.25 mm diameter; S6 2.72 mm thick, with a 0.8 mm diameter; S7 2.75 mm thick, with a 1.25 mm pore diameter; and S8 3 mm thick, with a 1.25 mm diameter.

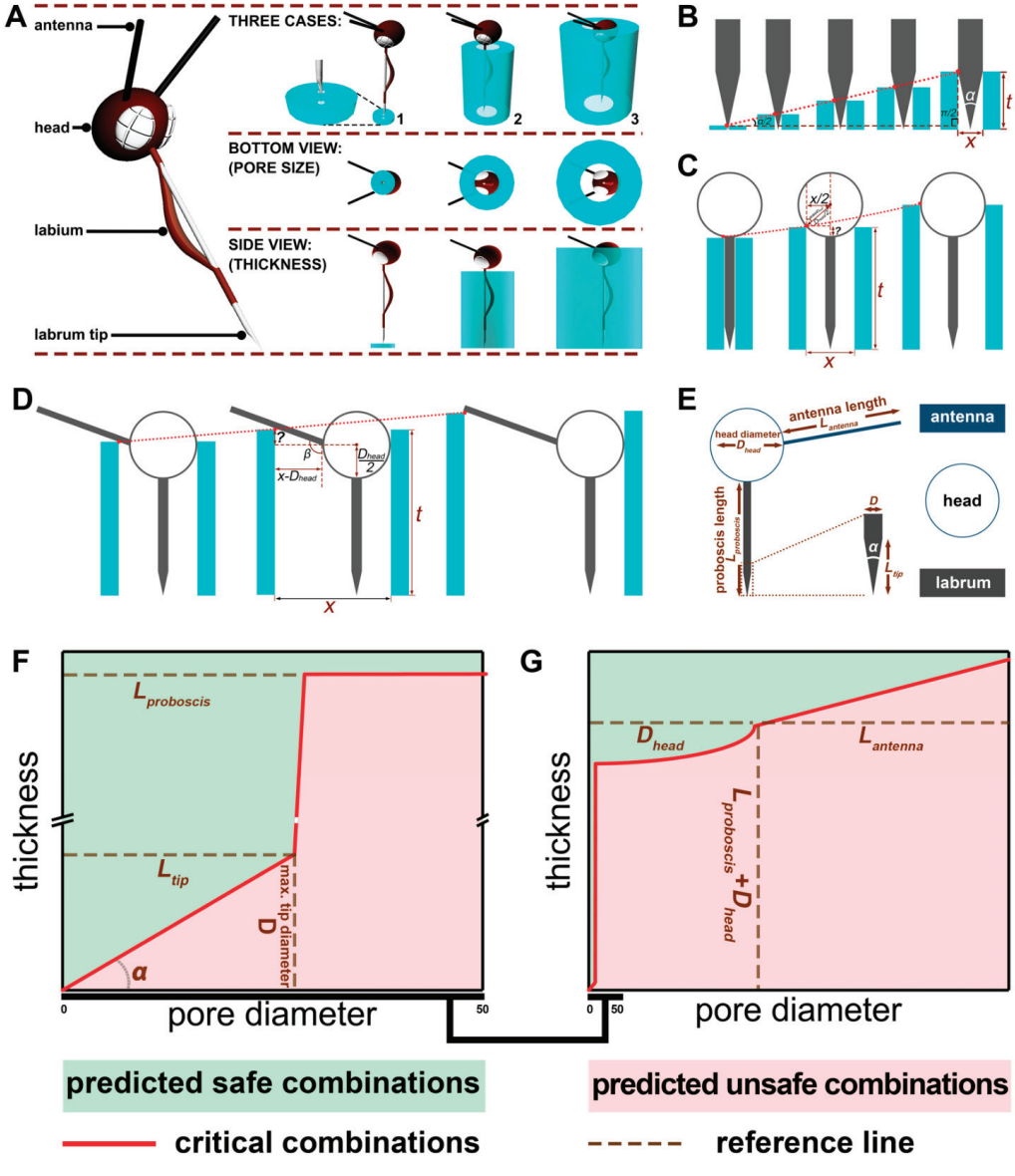

Figure 2. Bite-resistance model development. (**A**–**D**) Ice green vertical bars are the textile barrier, and the red dotted line the critical combination of pore diameter and thickness of the textile barrier. (**A**) Three cases that prevent mosquito biting based on mosquito anatomy. (**B**) Case 1—the pore diameter is smaller than the labrum diameter. (**C**) Case 2—the pore diameter is larger than the labrum diameter but smaller than the head diameter. (**D**) Case 3—the pore diameter is larger than head diameter but smaller than the sum of the head diameter and antenna length. (**E**) Abbreviations for length and diameter of the mosquito anatomy. (**F**) Zoomed-in view of the Case 1 model. (**G**) Case 1, Case 2, and Case 3 model predictions. Brown dotted lines in (**F**,**G**) are the critical parameters measured from the anatomy of the *Ae. aegypti* female in Figure S1 that define the three cases' combinations of porosities and thicknesses of the textile.

The holes in each plate were of uniform diameter. The ratio of open space (from the pores) to closed space (from the solid surface) was held constant in these studies. If the number of pores per plate was held constant but pore diameter increased, there would be an increasing probability that the probing mosquitoes would encounter a pore by chance alone. Furthermore, differences in the open area across a plate affects the amount of mosquito attractants (heat and odor [23]) penetrating through the holes in the plate. These attractants can affect landing and biting rates. Accordingly, as pore diameter was increased, a smaller number of pores were needed per plate. If the number of pores is designated as N and the diameter of a pore is designated as d with a unit of cm, the percentage of open area in a spacer should be a constant C, as shown in Equation (1):

$$C = \frac{N \cdot \pi (d/2)^2}{14.5 \times 3.4} \quad (1)$$

To keep the probability of a mosquito encountering a pore constant, the equation shows that the number of pores N in a spacer is inversely proportional to the square of the diameter of a pore, d. From the equation, the value of N was 572, 1396, and 3574 for pore diameters at 1.25, 0.8, and 0.5 mm, respectively.

For the Case 1 barriers, constructing thin plastic plates of ~75 μm or less by 3D printing was not possible. The thickness was too variable across the area of the plate. Furthermore, drilling small pores of ~28 μm or less by drilling across a thin plastic plate was not possible. To achieve the operational parameters needed to test the Case 1 model, commercially available Saatifil® polyester woven filtration fabrics were used (W = woven; W1, W2, W3, and W4, listed in Table 1) (shown in Figure S2B). In Figure S5A,B, W1 is 52 μm thick with a 25 μm pore dimeter, W2 is 60 μm thick with an 18 μm diameter, W3 is 58 μm thick with 14 μm pores, and W4 is 86 μm thick with 8 μm pores. These fabrics had square pores produced when the polypropylene monofilaments were woven in a plain weave pattern. The size of each woven fabric was 14.5 cm × 3.4 cm to fit into the *in vitro* bioassay device already described. We evaluated the bite resistance of four monofilament woven fabrics and the plastic blocks using the *in vitro* bioassay described earlier.

2.7.2. Knitted Textile Structures

To further validate our model for flexible textiles (T = textile materials; Table 1), we constructed fabrics including one predicted unsafe and one predicted safe according the model for each Case.

Case 1: The Case 1 fabric (T1; Figure S2D) was an ultra-fine synthetic knit of 80 percent polyamide of 20 denier count (a unit of measure for the linear mass density of fibers, the mass in grams per 9000 m of the fiber) and 20 percent elastane of 15 denier count and has a weight of 82 g/m^2. Its pattern was a jersey plated knit structure of 78 wales and 104 courses per inch and with a pore size between 32 and 42 μm. The pore diameter of T1 in Figure S5F was larger than the diameter of the mosquito labrum. To reduce the pore diameter based on our Case 1 model, we used a 1 m-wide, laboratory oil-heated Stork laminator (Stork GmbH, Bavaria, Germany) to heat set the fabric in the Dyeing and Finishing Pilot Plant at NC State University. The temperature was 190 °C (lower than T_g of the polyamide) with a 120 s duration. It was found that the pore diameters of the fabric (T2) was reduced by this treatment to 10 μm from 16 μm and the thickness reduced to 0.26 mm, as shown in Figure S5E,F (predicted to be safe by the Case 1 model).

Case 2: 3D spacer fabrics (T3, T4: satin weave + pillar stitch; Figure S2E) were produced on a double-needle bed, Raschel warp knitting machine with six guide bars (Rius Mini-tronic Raschel Warp Knitting Machine, RIUS-COMATEX, Barcelona, Spain) in the Knitting Laboratory at the Wilson College of Textiles at NC State University. The material consisted of 100% polyester (Huizhou City Meilin Textile Co., Ltd., Huizhou, China). For the pile yarn, a 33 dtex (a unit of direct measure of yarn linear density, grams per 10 km of yarn) monofilament was used. The outside surface was made with 55 dtex multi-filaments. Both multi-filaments contained 36 filaments, respectively. To make variations in the design,

the take-up speed was changed. Hence, the stitches per cm and the thickness would change. The T3 fabric was made by a 700% take-up speed, and the T4 one made by a 900% take-up speed. The combination of thickness and pore diameter of the T3 (Figure S5E,F) was predicted unsafe while that of T4 was predicted safe.

Case 3: The 3D spacer (warp) knit fabric for Case 3 had the same pattern and materials as the Case 2 fabrics, which were produced on the same Raschel warp knitting machine. Case 3 fabrics T5 and T6 (Figure S2E) were produced at 1500% and 1200% take-up speeds. The T5 thickness was 2 mm with a pore diameter of 940 μm. T6 was 3 mm and 770 μm, respectively (Figure S5E,F). Based on the model prediction, T6 is a safe material that should resist mosquito bites.

We evaluated the bite resistance of the Case 1, Case 2, and Case 3 fabrics using the *in vitro* bioassay system described earlier. All the materials used in the model validation, as listed in Table 1, including the woven textiles, plastic plates, and knits, were white in color to avoid potential mosquito preferences in landing and biting based on color differences.

2.8. Finite Element Model for Proboscis Penetration

In addition to our Case 1–3 conditions, we needed to investigate the point of contact of the proboscis to a textile surface and how this specific interaction might impact our prediction of penetration (especially relative to the Case 1 model). The finite analysis model was necessary because for Case 1, predictions based on labrum diameter alone were not 100% correct in predicting blood feeding when approaching the boundary between safe and unsafe textiles (Figure 3B). This result suggested additional physical interactions might be in play that were important in preventing biting. Finite Element Analysis was conducted for a woven versus a knitted structure to examine two possible scenarios for micro-deformation. The woven model was used for investigating the interaction of the woven structures and the knit to understand the role of stretching.

Structural parameters of woven and knit structures were obtained by the calculation of fabric thickness, weave density and spatial axial distribution [11,24], which were then imported into SolidWorks®, a computer-aided design program, for establishment of a geometrical model. The boundary conditions of both the woven and knit model were set to periodical boundary conditions [25] for approximating a large (infinite) fabric piece by using a small fraction of the piece. Since only a small force is applied in both scenarios, the mechanical property for the knit and woven model can be treated as linear elastic materials.

To simulate the pore deformation of the woven structure, a virtual labrum with the same mechanical properties and shape of a real mosquito labrum was used to penetrate the woven fabric. This virtual labrum will be discussed more later. The test was analyzed using the software suite SIMULIA Abaqus/Explicit 6.14. The elastic modulus and Poisson's ratio of the polyester monofilament used in this model were 2.16 GPa and 0.3, respectively.

For modelling the virtual labrum, we needed the fundamental mechanical properties of the proboscis. Because of its small size, traditional methods to measure tensile and compression [26] were not possible. Alternatively, the elastic properties of the proboscis were determined with a Bruker Hysitron TI980 Triboindenter (in the NC State University Analytical Instrumentation Facility). The measured location and load–depth curves are shown in Figure S7C. The elastic modulus of the proboscis can be achieved by the initial part of the recovery curve [27].

To simulate the pore deformation of the knit structure, virtual tensile forces were applied to the model in the course and wale directions (Figure S7B), and the simulated deformations compared with the real fabric deformation (Figure S7D,E). The elastic modulus and Poisson's ratio of the blended yarn used in this model were 1.08 GPa and 0.21, respectively. The knit model was validated using the experimental tensile data (Figure S7C) to ensure they have an equivalent mechanical property as the real knit fabrics.

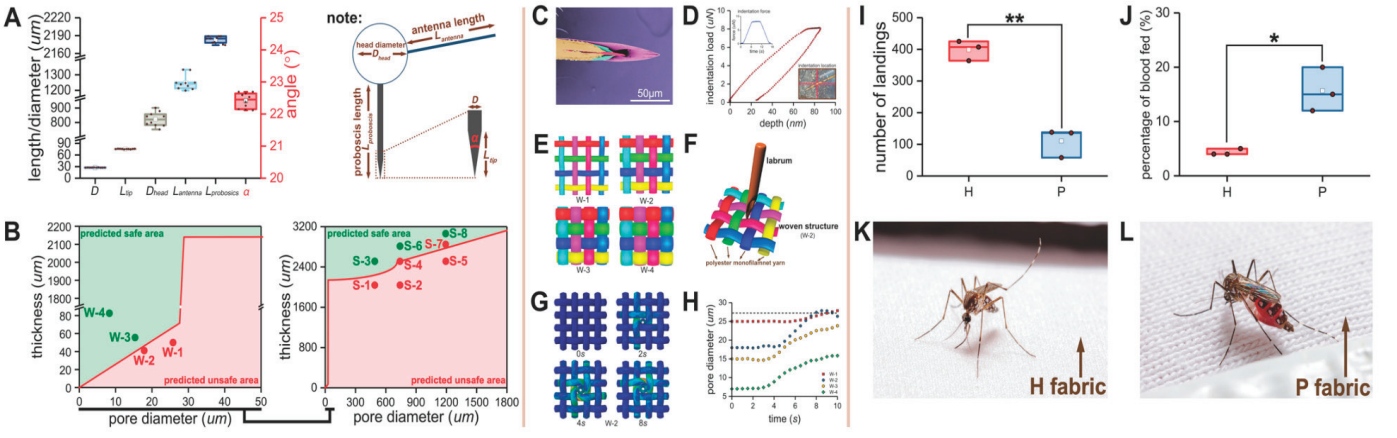

Figure 3. Mosquito morphometrics, model prediction based on mosquito morphometrics, impact of fabric distortion on biting, and comparison of non-insecticide versus insecticide-treated textiles for bite resistance. (**A**) Measured parameters of mosquito anatomy (average value calculated from 20 measurements for each parameter). (**B**) Model prediction of safe and unsafe woven filtration fabrics (left graph) and plastic plates (right graph). See Figure 4 and Table 1 for the in vitro bioassay results and Table 1 for the barrier abbreviations and whether the prediction was correct. (**C**–**H**) A demonstration of the textile structure failing to resist the mosquito bite at the critical boundary between safe and unsafe (Figure 3B) due to enlargement of the pore under labrum penetration. (**C**) Tip of proboscis. To measure the resistance to proboscis penetration, the mechanical property of the labrum (pink color) was measured. (**D**) Nanoindentation curve of the labrum. The elastic modulus was 1.35 GPa calculated by the load–depth curve. (**E**) Illustration of four weave patterns (W = Case 1 validating Woven structures, W1 to W4). (**F**) Model of the W2 fabric under pressure from proboscis penetration. The elastic modulus and Poisson's ratio of the polyester monofilament used in this model was 2.16 GPa and 0.3, respectively, evaluated on the MTS® tensile tester with 2 cm gauge and 5 mm/min speed. (**G**) Deformation process of W-2 subjected to proboscis penetration. (**H**) Variation of the pore diameter caused by proboscis penetration. The black dashed line is the maximum proboscis diameter. Pore diameters of W1 and W2 increased beyond this critical value, and thereby failed to resist proboscis penetration. (**I**,**J**) Arm-in-cage bioassay results for fabrics H and P: (**I**) difference in the number of landings was statistically significant at $p < 0.01$; (**J**) difference in the percentages of blood-fed mosquitoes was significant at $p < 0.05$. (**K**) Mosquitos failing to probe through the H fabric because of its small pore size (obvious proboscis bending trying to push through the H fabric). (**L**) Blood-fed female after successfully penetrating through the P fabric.

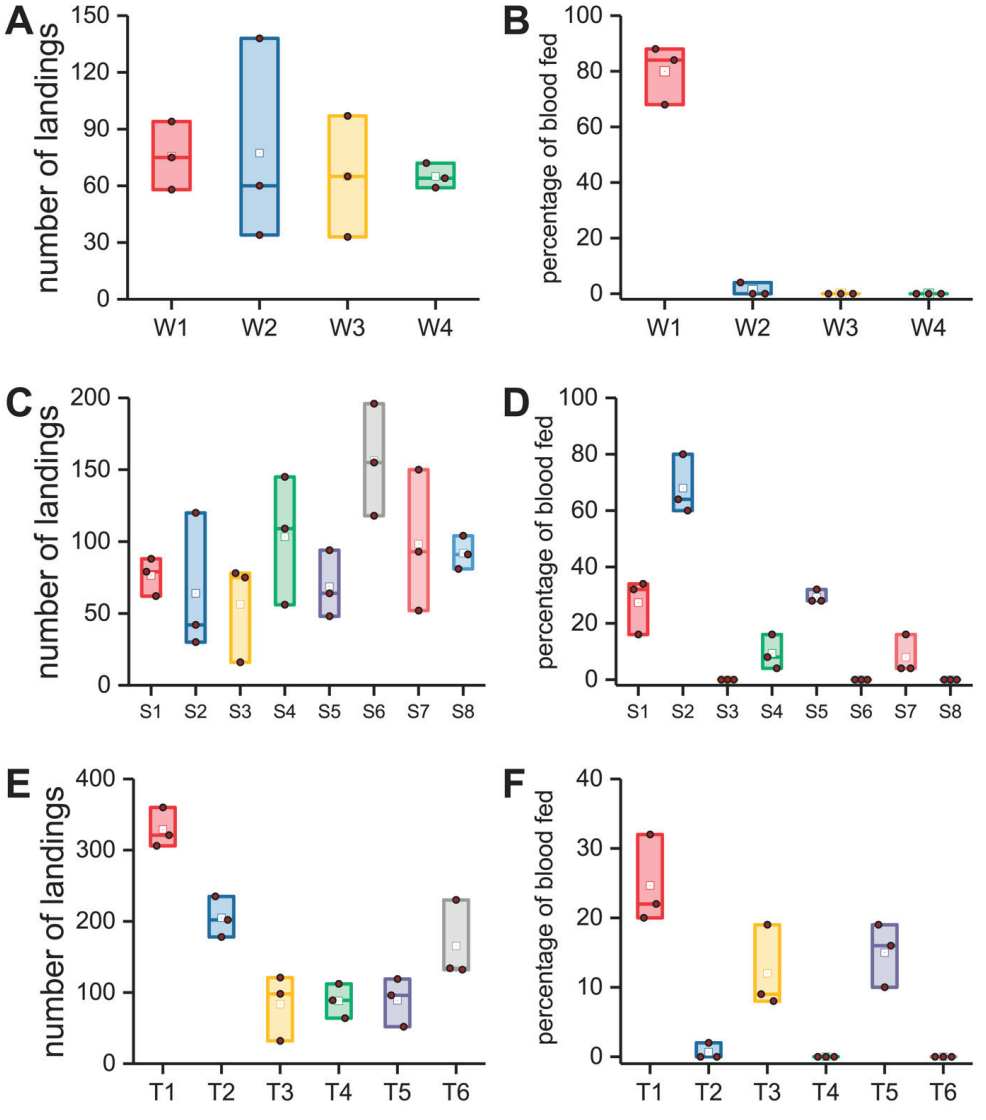

Figure 4. In vitro bioassay results for the woven structures, plastic plates, and knitted and knitted spacer fabrics (see Table 1 for the pore size and thickness, model prediction, and whether the prediction was accurate; Figure 3B for the position of the woven structures and plastic plates relative to the safe and unsafe barriers predicted by the model). (**A**) Number of landings on the woven structures. (**B**) Percentage of blood fed on by mosquitoes on woven structures. (**C**) Number of landings on plastic plates. (**D**) Percentage of blood fed on by mosquitoes on plastic plates. (**E**) Number of landings on knitted fabrics. (**F**) Percentage of blood fed on by mosquitoes on knitted fabrics. Abbreviations used: W1 to W4 = Case 1 woven filtration structures; S1 to S8 = Cases 2 and 3 plastic spacer blocks; T1 to T6 = Cases 1–3 knitted and spacer (3D) knitted textiles (see Table 1 for more detailed definitions of the abbreviations).

2.9. Prototype Bite-Resistant Fabrics Tested for Garment Construction

Three knitted fabrics (H, B, S; Table 1 and Figure S3B–D) were developed as component textiles for garment construction. They were selected from a dataset of candidate bite resistant fabrics that were predicted safe by our bite-resistance model. These textiles were assayed using arm-in-cage bioassays since the goal later was to test them in garments on human subjects in walk-in-cage studies.

Case 1 H. The Case 1 fabric H (the high-density fabric, H; Figure S3B) was an ultra-fine synthetic knit of 80 percent polyamide of 20 denier count and 20 percent elastane of 20 denier count and had a weight of 96 g/m^2. Its pattern is a jersey plated knit structure of 84 wales and 112 courses per inch and with a pore size between 20 μm and 28 μm, allowing air passage but preventing mosquito biting. It had a high elasticity of 400% stretch in the course direction and 160% stretch in the wale direction (Figure S7C). The H fabric has a more elastane content and smaller pore size compared with T1, which came from the same knitting technology. It was made into a base layer in the following section "construction of protective garments". Although the H fabric was not a 100% bite-resistant material due to an irregular pore distribution in the knit pattern, when combined as a base layer with military issued garments, a 100% bite resistance was possible in whole-garment testing.

Case 1 B. Fabric B (a bonded fabric; Figure S3C) is the combination of two layers of H fabric that was made by applying a small dot pattern of dry low-melt adhesive (CG-1698 polyurethane adhesive, Chemix Guru Ltd., Taichung, Taiwan) to one surface and then feeding the two fabrics back-to-back together applying pressure using heated drums (temperature 120 °C, duration 20 s). The two fabrics are fused together at regular intervals, and then the adhesive dots subjected to cool circulating air for 24 h to eliminate volatiles that might affect mosquito biting. The paste dot application procedure is particularly gentle to the substrate, and the wide range of options for formulating the paste provides the user flexibility in the application procedure. The relative nature, drape, porosity, and flexibility of the fabric is maintained, and this method only adds approximately 5% to the total weight. The B fabric is highly stretchable and demonstrated high mosquito bite resistance, which makes it suitable to being used as an outer protective garment.

Case 2 S. The S fabric (3D spacer fabric; Figure S3D) was a commercially available 3D warp knit spacer fabric (Production ID: 34836, Springs Creative Products Group, LLC, Rock Hill, SC, USA) that was predicted safe for bite protection using our Case 2 model. The surface (top and bottom) yarns are PA filament tows, and the pile yarns used in the middle layer were PA monofilaments. The surface patterns are shown in Figure S3D. The S fabric had a stable structure with large openings outside that allowed air flow into and under the garment, thereby transporting of heat and sweat out.

Case 3. Case 3 fabrics were translucent due to their large pores and not practical when used alone for typical garments where human body parts need to be covered and not seen by others. Therefore, we did not use the Case 3 fabrics to assemble a garment. This is not to say this fabric does not have uses for mosquito protection in parts of the body where it is ok to show the skin or as a cover at the beach or in the tropics where there are mosquitoes and also high thermal challenges to the body. The materials could also have uses for garment ventilation in specific areas of a garment.

Base on the color requirement for military garments, the H fabric was dyed to a light brown color before assembly into the base layer. B and S were dyed to a camo color before assembly into the military-style shirt (NCSU shirt).

2.10. Textile Structural Analysis

As mentioned before, fabric pore size and thickness are two critical factors in our model that determined bite resistance. Hence, it was important to measure these variables accurately. Pore areas in textile materials, especially in knitted fabrics, have irregular shapes due to complex fiber configurations. Pores with an elliptical shape often failed to resist mosquito bites even though the pore openings were narrower than the proboscis in one direction. We also found irregular pore openings were difficult to measure accurately

and were not informative to our model. Therefore, we assumed pores to be circular, and we measured pore diameter across the widest area of fabric pores so that the model would reflect a worst-case scenario.

Pore diameter was measured (Figure S4) with a digital microscope (Bausch & Lomb, Monozoom-7 Zoom Microscope), and images analyzed using ImageJ software, an open-source image-processing program designed for analyzing multidimensional images [28]. Based on Feret's diameter, the width of the pore along its longest direction, a frequency distribution of the pore diameters, and a fitting curve were obtained. From the peak of the fitting frequency distribution, we picked three maximum diameters for each fabric to calculate the average maximum pore diameter (4 images were captured for each fabric, a total of 12 measured values). Fabric thickness, measured with a Thwing-Albert ProGage Thickness Tester (Thwing-Albert ProGage instrument company, West Berlin, NJ, USA) was averaged over 10 tests, using standard methods for assessing textile thickness, as described in the ASTM D1777 guidelines [29]. The procedure of measuring pore diameter is shown in Figure S4, and the values of the measured pore diameters and fabric thicknesses are shown in Figure S5.

2.11. Comparison of the Non-Insecticide and Insecticide-Treated Textiles

Before garment construction, it was prudent to understand how our bite-resistant, non-insecticidal textiles performed relative to a leading brand of insecticide-treated cloth. We compared the bite resistance of the H fabric with a commercially available permethrin-treated T-shirt fabric (P = permethrin, listed in Table 1), which was cut from an InsectShield® T-shirt (RN149846, Insect Shield, LLC, Greensboro, NC, USA) purchased from a local retail store. The fabric was 70% cotton and 30% polyester and cut into 14.5 cm × 3.4 cm for the arm-in-cage (in vivo) bioassays.

2.12. Construction of Protective Garments

Based on the predictions of our model, three types of fabrics were used as bite resistant materials: a superfine knit fabric (H), a double-layer bonded knit fabric (B), and a knitted 3D spacer fabric (S), as shown in Figure S3B–D. Two types of garments were produced: a base layer and a military-style combat shirt, as shown in Figure S6A,B.

Base layer (Figure S6A). A form-fitting undergarment was constructed consisting of an upper body, form-fitting garment having a torso section and arm sections made from the Case 1 fabric H. The garment was fitted with an elastic neck cuff secured to define a neck opening for the torso section; an elastic waist cuff secured to define a waistband around the torso section; and a pair of elastic wrist cuffs disposed at an outer terminus of each of the arm sections. The ensemble also included a lower-body, form-fitting garment having a waist section and left- and right-leg sections made from the same textiles as previously described for the shirt. The pants were fitted with an elastic waist cuff secured to define the waistband around the waist section and a pair of elastic ankle cuffs disposed at the terminus of each of the left and right leg sections. The cut and sewing of this garment were conducted in the Fashion Studio at the Wilson College of Textiles at NC State University. The garment was unwashed and tested in walk-in-cage studies (described earlier).

NCSU shirt (Figure S6B). A long sleeve shirt was constructed as an upper-body, form-fitting garment. The shirt consisted of Case 1 B and Case 2 S fabrics. The incorporation of the B fabric provides extensionality and bite resistance, while the use of the S fabric brings breathability, pressure release, and bite resistance to the shirt. The S fabric was designed into the sections of the shoulders, chest, back, and elbow of the garment, and the remainder of the shirt was the B fabric. The cut and sewing for this garment were conducted in the Fashion Studio at the Wilson College of Textiles at NC State University. The garment was unwashed and tested in walk-in-cage studies (described earlier).

Both garments were sewed on an MF 7924 cover stitch sewing machine (JUKI, Singapore) and locked on a DDL-8700-7 lockstitch machine (JUKI, Singapore). The sewing thread was 100% polyester (RCL, model: RCLJ-ST-W, Wuxi, China). The seams were bite

resistant in the walk-in-cage bioassay, since there was a two-layer overlap of the textile at the connections between the two pieces of cloth.

2.13. Sweat Manikin Test for Comfort Evaluation of Garments

In the Textile Protection and Comfort Center of NC State University, a sweating manikin was used to evaluate the thermal insulation and breathability of the garments [30] (Figure S6D). The test instrument is composed of a manikin, an environmental chamber, an ambient detector, a power supplier, a water reservoir, and a pump.

Comparisons were made with a commercially available base layer garment (Under Armour® men's base 1.0 crew, model: 1281079, Under Armour Inc., Baltimore, MD, USA) and a military-issued combat shirt (Winter Army Combat Shirt Test, made in the USA by NIB/NCW, Figure 5A). The comparison garments had similar material characteristics and knit patterns to our garments. Each comfort evaluation was replicated three times, after which average values were calculated.

Manikin zones (a group of thermal-sweat elements on the manikin) were measured for thermal resistance and evaporative resistance. The standard method for measuring thermal resistance is described in ASTM F1291 and was followed. Test conditions for thermal resistance were 20 °C, 50% relative humidity, and a 0.4 m/s air speed with a 35 °C skin temperature. The measurement standard of evaporative resistance was ASTM F2370. Test conditions for evaporative resistance were 35 °C, 40% relative humidity, and a 0.4 m/s air speed with a 35 °C skin temperature. The following parameters were obtained from the manikin test: R_t (°C·m^2/W), the total thermal resistance provided by the manikin, garment ensembles, and air layer; R_{et} (kPa·m^2/W), the total evaporative resistance provided by the manikin, garment ensembles, and air layer; R_{cl} (°C·m^2/W), the instinct thermal resistance provided by the garment ensembles only; R_{ecl} (kPa·m^2/W), the instinct evaporative resistance provided by the garment ensembles only; I_t (clo), the total insulation provided by the manikin, garment ensembles, and air layer (higher I_t values mean the garment has a higher thermal insulation property that would not be desirable in warm weather for a bite-resistant fabric); i_m, the moisture-heat permeability through the fabric on a scale of 0 (total impermeable) to 1 (total permeable) normalized by the permeability of still air on the naked skin; and $Q_{predicted}$ (W/m^2), the predicted heat loss potential, which gives a predicted level of the total amount of heat that could be transferred from the manikin to the ambient environment for a specified condition. The $Q_{predicted}$ incorporates thermal and evaporative resistance values to calculate the predicted levels of evaporative and dry heat transfer components for a specific environmental condition. In this case, the specified environment was 25 °C and a 65% relative humidity. The overall $Q_{predicted}$ under these conditions was calculated by adding the predicted dry component of heat loss to the predicted evaporative component of heat loss and reflected the predicted total amount of heat loss possible. The test results of all parameters are shown in Table S1.

2.14. Data Analysis

All the replicated data for the assays and comfort analyses (Figures 3–5 and Figure S5) were plotted in ORIGINPRO® 2018 using a box plot format, a graphical format that summarizes the key statistical values. The solid brown dot in the box plot was the raw data. The height of the box represents the 25th and 75th percentiles. The whispers represent the 5th and 95th percentiles. Additional values included the median (line inside of the box) and mean (white dot) presented in the box plot. We used the mean value of each data set for our analyses.

We used one-sample Student's *t*-tests to investigate the significance between two data sets in Figures 3I,J and 5B,C The mean value of the first data set was used as the theoretical expectation. The second data set was set as the true mean. Differences in mean values were found to be statistically significant when the *p* values were greater than 0.05 (*) or 0.01 (**).

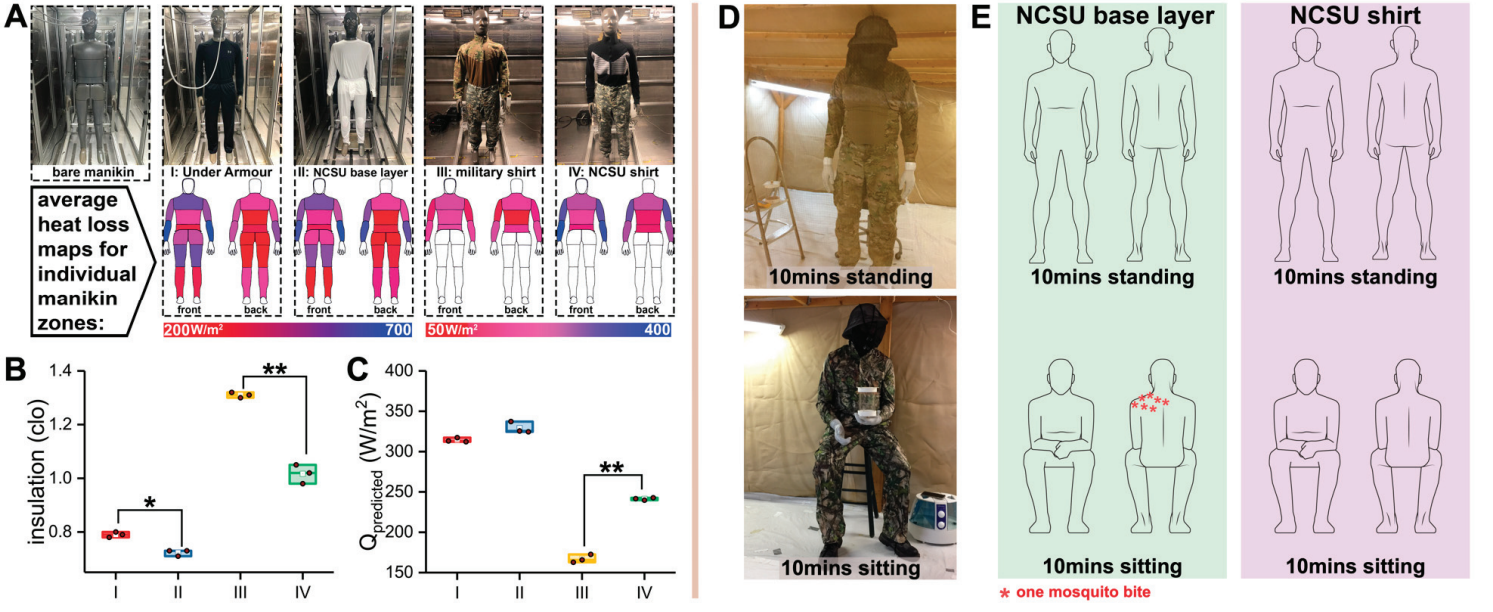

Figure 5. Prototype garment's comfort and bite-resistance compared to commercially available similar garments. (**A**) Manikins equipped with various garments (I, Under Armour® base layer; II, NC State base layer; III, winter army combat shirt; and IV, NC State shirt), also showing the average heat-loss maps. (**B**) Garment insulation. Since mosquitoes mostly appear in warm weather, a garment with low insulation properties is preferred. The NCSU base layer and NCSU shirt provided lower levels of insulation compared with the comparative garments tested ($p < 0.05$ and $p < 0.01$, respectively). (**C**) The $D_{predicted}$ values (predicted heat loss; Table S1) for the garments tested. II the NCSU base layer showed an equivalent thermal and moisture management compared with I. IV, the NCSU shirt exhibited better thermal and moisture management compared to III ($p < 0.01$). (**D**) Walk-in-cage bioassay with 10 min standing and 10 min sitting. The container in the hands of the subject (bottom picture) housed the mosquitoes. The mosquitoes were typically released, and the test started with the person standing (note the empty container on the stool, top picture). (**E**) Walk-in-cage bioassay results for the worst-case replicate shown (* = one mosquito bite). Bites on the shoulder were observed where the most stretching of the garment occurred and bite resistance was reduced. A specially designed double layer was used in this part of the NCSU base layer which eliminated all bites in the walk-in-cage bioassay (data not shown).

All tested materials and garments are listed in Table 1, including information on the material type, name, abbreviation, thickness, pore diameter, model prediction, and bioassay validation. Values of thicknesses and pore diameters are the mean values calculated from the multiple measurements discussed in the section "Textile structure analysis". Model prediction is the predicted bite resistance. "Safe" represents a fabric that is predicted to have 100% bite protection predicted by the bite-resistance model and "unsafe" means the fabric is predicted to allow at least 1 mosquito bite. Bioassay results are actual measurements of bite resistance. "Pass" indicates the fabric was at least 95% bite resistant by the in vitro or in vivo bioassay. "Fail" indicates a fabric provided less than 95% bite protection.

3. Results and Discussions

3.1. Mosquito-Bite-Resistant Textile Model

Figure 1A shows an adult female *Ae. aegypti* probing human skin. Figure 1B is a scanning electron microscopy picture (SEM) of a knitted textile. The yarns used to make the textile consisted of a multitude of filament fibers knitted in an intermeshed loop configuration. In a knitted fabric, the spaces between the filaments form pores (Figure 1C) and together with its thickness determine a fabric's bite resistance to mosquitoes and its comfort to people. Pore diameter and fabric thickness are critical limiting factors for mosquito proboscis penetration of the skin that also affect the thermophysiological comfort of a textile (Figure 1D). Increasing pore diameter improves fabric breathability and comfort but increases the transmission of skin odorants, increasing mosquito landings and biting. Fabrics containing small pores are less attractive to mosquitoes and more bite resistant but have reduced comfort because of reduced air flow. Increasing fabric thickness improves bite resistance but reduces comfort by increasing thermal insulation. A model to predict bite resistance was developed that informed fabric thickness and pore diameter as they related to the morphometrics of the mosquito's head, antennae, and proboscis, and the mechanism that mosquitoes use for finding and biting through a textile. The three cases considered are illustrated in Figure 1E. Figure 1F describes our overall strategy for developing bite-resistant garments: (i) developing a predictive model based on mosquito head morphometrics; (ii) model validation using mosquito *in vitro* testing of woven filter fabrics, plastic spacers, and 3D spacer fabrics for bite resistance; (iii) development of knitted fabrics for garment construction using the model; (iv) *in vivo* (arm in cage) mosquito testing for bite resistance of these fabrics; (v) garment construction; and (vi) garment walk-in-cage testing for bite resistance; and (vii) manikin comfort tests of the garments.

Figure S1A shows the size of the proboscis where the stylets of the proboscis interlock forming a feeding tube covered by the labium (Figure S1B). Figure S1C shows the stylets, and Figure S1D is an SEM of the mosquito's proboscis composed of the labrum, maxillae, mandibles, and hypopharynx. The mechanical process of probing skin was described previously [31,32]. The labrum's diameter was measured in our work as a key parameter for our bite-resistance model. Preventing labrum contact with the skin prevents blood feeding. Figure 2A–D provide a detailed description of Cases 1–3. In Case 1, the pore diameter of the fabric barrier is smaller than the diameter of the labrum (Figure 2B). In Case 2, the pore size of the fabric barrier is larger than the labrum diameter but smaller than the diameter of the mosquito head (Figure 2C). Thus, fabrics with the proper thickness can prevent the labrum tip from contacting skin. In Case 3, the fabric pore size is larger than the head diameter but is smaller than the size of the head plus antennae (Figure 2D). The ice-green vertical bars are the textile barrier, and the red dotted line the critical combination of pore diameter and thickness of the textile barrier.

The critical geometrical relationships of pore diameter and thickness for each case to prevent blood feeding were defined as follows:

Case 1:

$$t = \frac{x}{2 \times tan\left(\frac{\alpha}{2}\right)},\ when\ 0 \leq x < D \tag{2}$$

$$\frac{D}{2 \times tan\left(\frac{\alpha}{2}\right)} \leq t \leq L_{proboscis},\ when\ x = D \tag{3}$$

Case 2:

$$t = L_{proboscis} + \frac{D_{head}}{2}\left\{1 - cos\left[arcsin\left(\frac{x}{D_{head}}\right)\right]\right\},\ when\ D < x \leq D_{head} \tag{4}$$

Case 3:

$$t = L_{proboscis} + \frac{D_{head}}{2} + \tan(\beta - 90) \times (x - D_{head}), when\ D_{head} < x \leq L_{antenna} \tag{5}$$

where t and x are the thickness and pore diameter of the mechanical barrier, respectively; $L_{\text{proboscis}}$ is the maximum proboscis length; D is the maximum diameter of the proboscis tip; α is the angle of insertion of the proboscis tip; and β is the angle between the antenna and proboscis.

The red dotted lines in Figure 2B–D show the limit between a textile being predicted as unsafe (biting is possible) and safe (biting cannot occur) for Cases 1–3 (for critical combinations of pore sizes and thicknesses as specified by the model). For the model to be feasible, we made the following assumptions: (1) the fabric barrier and proboscis tip were not deformable; and (2) only thickness and pore diameter were considered as structural parameters for the fabric barrier. Figure 2F,G show the correlation between the bite-resistance performance predicted by the model and fabric pore size and thickness, in which the abbreviations of all dimensional values are described in Figure 2E. In Figure 2F,G, the brown dotted lines mark the dimensions of the key factors of the mosquito anatomy, including the head diameter, labrum and its tip length, and diameter and antenna angle from the head and length. The red solid lines are the critical combinations of the fabric pore diameter and thicknesses relative to the mosquito morphometrics that would produce a safe (100% bite resistance shown in green) or unsafe (pink) fabric as predicted by the model.

3.2. Mosquito Morphometrics Used to Predict Safe Fabrics

The head diameter (D_{head}), antenna length (L_{antenna}), proboscis length ($L_{\text{proboscis}}$), maximum labrum diameter (D), labrum tip length (L_{tip}) and the tip angle (α) of *Ae. aegypti* adult females are shown in Figure 3A. Each body part was measured from twenty insects. The average values were input into our model to define the fabric thickness and pore diameter and the limit between safe and not safe (Figure 3B). We focused on these limits and produced a variety of barriers of different pore sizes and thicknesses for the experiments (Figure S5A–F) to test the model using our *in vitro* bioassay (Figure S2A). In some cases, these barriers (description follows) were not practical for garment construction but were used because they were optimum for model validation, as explained in the Materials and Methods.

For Case 1, single-filament (woven) filter fabrics (shown in Figure 1Fii and Figure S2B) with different pore sizes and a fixed thickness (Figure S5A,B) were tested using the *in vitro* mosquito-bite-resistance bioassay (Figure 1Fii and Figure S2A). These are technically fabrics, but they are highly resistant to stretch, uncomfortable to wear, and too costly for garment construction. However, they were used for model validation because they were available in precise, different pore diameters and fabric thickness. Highly precision-machined, polypropylene plastic plates (Figure 1Fii and Figure S2C) were used with different pore sizes and thicknesses (Figure S5C,D) to evaluate the model for Cases 2 and 3 using the *in vitro* bioassay. Then, two knit fabrics for Case 1 and two knitted spacer fabrics (shown in Figure 1Fii) for Cases 2 and 3 each with different pore diameters and fabric thickness (Figure S5E,F) were constructed to inform further on Cases 1–3, to better approximate a practical garment application than filter fabrics and plastic plates.

The number of landings and percentage blood feeding for the barriers tested are shown in Figure 4 for our model validation research. Table 1 (group = materials for model validation) relates thickness and pore diameter to the model prediction and whether the

barrier failed or passed in preventing mosquito blood feeding. In these experiments, a percentage of blood feeding greater than 5% (bite resistance was lower than 95%) was considered a failure for the barrier in preventing blood feeding. In Figure 3B, the left and right graphs relate the pore size and thickness for the filter fabrics and plastic plates, respectively, with the model prediction of what would be safe and unsafe. Only one (plastic plate S7, Table 1) out of the 18 barrier materials tested (filter fabrics, plastic plates and knit fabrics) failed to provide bite protection when the model informed the barrier should be safe. This failure in the model corresponds to the red dot in the green area in Figure 3B, the right graph. Those barriers (green color dots) located in the safe area exhibited bite resistance against mosquitoes of at least 95%, as the model predicted for the filter fabrics and plastic plates. The model was 100% accurate in predicting safe and unsafe for both the knit and knitted spacer fabrics (Table 1, T1–T6).

These results suggest that the model we developed was reliable for predicting mosquito-bite resistance against the lab-reared mosquito, *Ae. aegypti*, and was 100% reliable in our studies of the knits and spacer knits tested. Additional testing will be needed in the future, to determine if our model translates to other mosquito species and to mosquitoes in the field. Regarding for the economy of time and resources, we argue concentrating on one species was a reasonable approach for our studies and proof of concept.

3.3. Finite Element Analysis

In our validation studies, a barrier was considered safe when bite resistance was 95% or higher. When pore sizes and thickness approached the limit between safe and unsafe (Figure 3B left graph for filter fabrics and right graph for plastic plates), some blood feeding occurred at a low percentage, 5% or less (Figure 4A–D). This was also the case for the knits tested (Figure 4E,F). There are two possible reasons. First, the labrum diameter of some mosquitoes may have been smaller than the average value (27.5 μm) used in the model, allowing some mosquitoes to penetrate the barriers. Second, the barrier may have deformed under the pressure of the proboscis and enlarged the pores causing failures. In the latter case, this would not be an issue with the plastic plates but could be a factor for the textiles tested.

To investigate the interaction between proboscis and textile structure, the elastic modulus and geometry of the labrum were measured to establish a finite element labrum model. Figure 3C shows the anatomy of the proboscis tip. Figure 3D is the nanoindentation curve for the labrum, which was used to obtain the elastic modulus for the property parameters needed for the model. The woven (filter) fabric used in our validation studies (Section 3.2), W1 to W4 (Table 1), were modeled to better understand how the labrum might deform textiles in general. Figure 3E illustrates the four patterns. Figure 3F shows one example of the penetration model for the labrum on the W2 woven fabric, and Figure 3G shows the time course of penetration. For W1, the labrum interaction with the textile is less since the labrum can easily go through the fabric. However, W3 and W4 in Figure 3E are more dense structures with the pore size below that of the labrum diameter, not allowing free labrum penetration through the pore. Therefore, W2 with a pore diameter of 18 um was selected to show fabric deformation subjected to labrum penetration. It was found in our research that the labrum can move the filament yarn and push through the W2 filter fabric over time (Figure 3G) for a blood meal. This is the reason that W2 located near the boundary line failed in resisting some mosquito bites. Figure 3L shows the change curves for the pore diameters of each woven structure. After labrum penetration, W1 and W2 were enlarged more than the labrum diameter and therefore would fail in preventing blood feeding because the structures were deformed. Although pores on W3 and W4 demonstrated deformation, the pore diameter was still below the labrum diameter, which enabled the structure to prevent blood feeding. In summary, in addition to the importance of pore size and thickness, the finite element analysis informs that micromechanical deformation of the fabric in response to the pressure exerted by the proboscis pushing-through the fabric can affect blood-feeding success. Yarn chemistry and methods of weaving and knitting will

impact deformation and, therefore, bite resistance. It would also be expected that variation in labrum diameter in the mosquito population will have an impact.

3.4. Development of Fabrics for Garment Construction

Once the model was validated for Cases 1–3, textiles were developed for the construction of a garment for final proof of concept that non-insecticide clothing could be bite resistant to mosquitoes and also comfortable. For these studies, bite resistance was measured with arm-in-cage bioassays (Figure S3A) with a textile considered safe if the bite resistance was 95% or higher. For Case 1, the knitted fabrics were H and B (Table 1) and shown in Figure S3B,C, respectively, and in Figure 1Fiii. For Case 2, the knitted spacer fabric was S (Table 1) and shown in Figure S3D, front and back, and Figure 1Fiii. Thickness and pore diameters are shown in Figure S5G,H, respectively, and the model prediction and bioassay results are in Table 1. The model was correct in all cases (see group = fabrics used in garments) in successfully predicting bite resistance. Accordingly, these textiles were used for garment construction.

3.5. Bite Resistance of an Insecticide-Treated versus Non-Insecticidal Textile

Permethrin-treated textiles are a widely used technology to prevent mosquitoes from biting people. Permethrin exhibits mosquito contact toxicity but also spatial repellency. Figure 3I shows the number of landings on fabric P (a permethrin-treated commercial fabric; detail on pore size and thickness in Table 1), which was lower ($p < 0.01$) than that for fabric H, the non-insecticidal superfine knit. Fabric P demonstrated spatial repellency presumably because of permethrin in the cloth whereas fabric H did not. Fabric H had a higher number of landings because mosquitoes were not repelled and landed on the fabric repeatedly in attempts to find a suitable location to penetrate the fabric. High landings without bites indicated the fabric structure has breathability but with pores sufficiently small for high bite resistance. Figure 3J shows that the percentage of blood-fed mosquitoes in the arm-in-cage studies for fabric P was three times higher than fabric H ($p < 0.05$). Although fewer mosquitoes landed on fabric P, a larger percentage of the mosquitoes that landed were able to penetrate the fabric and obtain a blood meal. In contrast, fabric H with smaller pore diameters and no insecticides resisted mosquito bites at a higher level.

These studies demonstrated that high bite resistance across a textile can be achieved that far succeed one commercial permethrin-treated fabric under high biting pressures in an arm-in-cage bioassay. Higher landings with no spatial repellency on the insecticide-free cloth would be expected to reduce biting on uncovered skin, especially when the proportion of uncovered to covered skin is small; in this case, the mosquitoes are probing the cloth and not being pushed to unprotected skin. However, more detailed studies are needed to address how an insecticide-treated textile versus a non-insecticide-treated textile, such as fabric H, would protect uncovered areas of the body.

3.6. Comfort and Bioassay Evaluation of Prototype Garments

The final step in demonstrating the proof of concept that insecticide free textiles can be used to protect humans from mosquito blood feeding and at the same time be comfortable, was to construct garments with the knits that our model predicted would be safe (fabrics H, B, and S, Table 1). These fabrics were used to construct a protective undergarment (a base layer garment; NCSU base layer, Table 1, and shown in Figure S6A and Figure 1Fv) and shirt (NCSU combat shirt, Table 1 and shown in Figure S6B and Figure 1Fv). These garments were tested in walk-in-cage bioassays to evaluate the mosquito-bite resistance where the threshold for success was no bites. A sweating manikin test was conducted to create whole-body heat loss maps for fabrics in different body zones to understand the heat and moisture resistance properties of our mosquito-bite resistant garments compared to commercially available garments.

Garments were tested for heat loss using a sweating manikin (Figure 5A). The garments included I, an Under Armour base layer; II, the NC State base layer developed using our model; III, a US army-issued combat shirt (provided by the US DOD); and IV, the NC.

State-developed, next-generation combat shirt, using our model. The same style of garments had similar heat-loss maps (Figure 5A), which indicated equivalent levels of thermal management. In the maps for garments III and IV, the blue color of IV is darker than III due to an innovative design that incorporated a 3D spacer fabric (Figures S3D and S6B and Figure 1Fiii) predicted to be bite resistant by our model but with open pores into the chest and arms area for heat management (Figure 5A).

The insulation values for both of our developed garments (Figure 5B) were smaller than their counterparts of the same style. This finding indicated that the NC State base layer and the NC State combat shirt had favorable thermal exchange as well as minimal heat accumulation, making the garments more comfortable to wear in warm weather. The Predicted Heat Loss Potential ($Q_{predicted}$, W/m^2) is a projection of the total amount of heat that could be transferred from the manikin to the ambient environment for a given condition, which was calculated using thermal and evaporative resistance values (see details in Table S1). In this case, the $Q_{predicted}$ of garments II (NC State base layer) and IV (NC State combat shirt) exhibited higher values than their counterparts (Figure 5C), which indicated they possessed superior comfort performance in both thermal and moisture management.

The NC State base layer and the NC State combat shirt were tested in walk in cage bioassays under heavy mosquito biting pressure with the human subject standing and sitting for 10 min in each posture (Figure 5D,E). The NC State combat shirt provided 100% protection against mosquito bites. However, the human subject wearing the NC State base layer received bites on the back and shoulders and the level of overall average protection was 96.5% (7 bites per 200 mosquitoes). When the base layer is used as an undergarment under a uniform, protection would be 100% (data not shown). This result on biting in the test reported was attributed to deformation of the knitted fabric on the shoulders where the fabric stretched, increasing the pore diameter of the fabric. We measured the fabric length during standing and sitting. Fabric H was estimated to have a 9.47% increase in stretch from the standing to the sitting postures. We conducted a virtual tensile experiment using an FEA model to investigate the change in the pore diameter of fabric H (see details in Figure S7). The tensile behavior of the fabric showed a directionality of stretch in which the wale direction exhibited a smaller deformation compared with the course direction, as shown in Figure S7C. The pore diameter also exhibited directional deformations in the course and wale directions, as shown in Figure S7D,E. In order to improve the bite resistance, a double layer of fabric H was stacked on the shoulder area (yoke), which partially covered the back of the human subject. The stacked orientation for both layers were perpendicularly aligned with each other, which reduced the fabric deformation during sitting and movement; this treatment also misaligned the pores of both fabrics. This improved the garment's bite resistance and provided 100% bite protection in walk-in-cage bioassays. Our two final garments listed in Table 1 were 100% bite proof in walk-in-cage tests. Notably, when the base layer was used as an undergarment under a uniform, protection was 100% (data not shown). In summary, preventing human–vector contact is an effective way to protect people from mosquito bites as well as to eliminate the threat of mosquito-borne diseases. We developed a mathematical model to predict the bite resistance of non-insecticidal textile barriers. Our model was verified through in vitro bioassays, using woven fabrics, plastic spacer plates, and knitted and knitted spacer fabrics, which showed that the model could accurately predict the bite resistance of mechanical barriers. The model was then used to develop comfortable and wearable textiles for garments. When compared with a permethrin-treated fabric, our fabrics development for garments had a higher bite resistance with a predicted higher level of protection for exposed skin; however, the latter needs further study. Then, the prototype garments were constructed with these textiles. These garments exhibited superior comfort performance compared to similar commercial garments and 100% mosquito-bite resistance. Use of our

model in the future will facilitate development of other, highly effective and comfortable bite-resistant fabrics solely based on textile structure without the need for an insecticidal treatment to prevent mosquito biting, and thus can be used to produce mosquito-bite-proof clothing for everyday use.

4. Conclusions

Preventing human-vector contact is an effective way to protect people from mosquito bites as well as to eliminate the threat of mosquito-borne diseases. We developed a mathematical model to predict the bite-resistance of non-insecticidal textile barriers. Our model was verified through *in vitro* bioassays, using woven fabrics, plastic spacer plates and knitted and knitted spacer fabrics, which showed that the model could accurately predict bite-resistance of mechanical barriers. The model was then used to develop comfortable and wearable textiles for garments. When compared with permethrin-treated fabric, our fabrics development for garments had a higher bite-resistance with a predicted higher level of protection for exposed skin; the latter needs further study however. Then prototype garments were constructed with these textiles. These garments exhibited superior comfort performance compared to similar commercial garments and 100% mosquito bite-resistance. Use of our model in the future will facilitate development of other, highly effective and comfortable bite resistant fabrics solely based on textile structure without the need for an insecticidal treatment to prevent mosquito biting and can be used to produce mosquito bite proof clothing for everyday use.

Supplementary Materials: The following are available online at https://www.mdpi.com/2075-4450/12/7/636/s1. enclosed in attachment.

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