



## Article

# Multiple Pesticide Resistance in Rust-Red Flour Beetle (*Tribolium castaneum*, Herbst 1797) from Northern Nigeria Is Probably Driven by Metabolic Mechanisms

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**Abstract:** There is a severe lack of information about molecular mechanisms of pesticide resistance in the rust-red flour beetle, a major pest destroying grains and flour across Nigeria, hindering evidence-based control. Here, we identified to the species level three populations of the red flour beetle from Kano, Nigeria, as *Tribolium castaneum* (Herbst 1797) and investigated the mechanism driving their insecticide resistance. The IRAC susceptibility bioassays established cypermethrin resistance, with LC<sub>50</sub>s of 4.35–5.46 mg/mL in the three populations, NNFM, R/Zaki and Yankaba. DDT and malathion resistance were observed in NNFM, with LC<sub>50</sub>s of 15.32 mg/mL and 3.71 mg/mL, respectively. High susceptibility was observed towards dichlorvos in all three populations with LC<sub>50</sub>s of 0.17–0.35 mg/mL. The synergist bioassay with piperonylbutoxide significantly restored cypermethrin susceptibility, with mortality increasing almost threefold, from 24.8% obtained with 1.5 mg/mL of cypermethrin to 63.3% in the synergised group ( $p = 0.013$ ), suggesting a preeminent role of P450s. The two major knockdown resistance (*kdr*) mutations, T929I and L1014F, in the IIS4 and IIS6 fragments of the voltage-gated sodium channel were not detected in both cypermethrin-alive and cypermethrin-dead beetles, suggesting a lesser role of target-site insensitivity mechanisms. These findings highlight the need to explore alternative control tools for this pest and/or utilise synergists, such as piperonyl butoxide, as additional chemistries in pesticide formulations to improve their efficacy.

**Keywords:** red flour beetle; *Tribolium castaneum*; pesticides; insecticides; resistance; metabolic



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## 1. Introduction

In Nigeria, wheat is the third most consumed grain after corn and rice [1], with a rise in consumption from 300,000 tonnes in 1970 to about 6 million tonnes as of 2021 [2]. Nigeria imports 98% of its wheat needs; in 2020 alone, \$2.15 billion was spent on wheat importation, making it the third most imported product in Nigeria and the country the fourth largest importer of wheat in the world [<https://oec.world/en/profile/bilateral-product/wheat/reporter/nga> (accessed on 12 January 2023)]. In Nigeria, wheat is milled into flour for conventional bakery products such as bread, cakes and biscuits, as well as making pasta, with the top companies—the Flour Mills of Nigeria Plc, Olam, Dangote, Chorghoury and Honeywell—milling 32%, 24%, 19%, 11% and 10% of the flour market share, respectively, while the rest of the other companies mill only 4% [1].

One of the major challenges for storage of milled flour is infestation by pests, such as the rust-red flour beetle, which in Nigeria is known to destroy/contaminate all the important cereals and their flour, e.g., maize (*Zea mays*), sorghum (*Sorghum bicolor*), wheat (*Triticum aestivum*), rice (*Oryza sativa*) and millet (*Pennisetum glaucum*) [3,4]. Indeed, this insect is a serious, cosmopolitan pest of stored grains and grain products in tropical and subtropical regions of the world [5], shown to destroy not only maize, wheat and other granaries, but also rice bran, oilseed, oil cake, nuts, dried fruit, spices, chocolate and even the bones of animals [6].

Infestation by the rust-red flour beetle results in the loss of quality and quantity of food products [4,7,8], as well as bad smells and tastes imparted to the food materials from the beetle's excrement [8] which contains pungent benzoquinones [9]. Several studies in Nigeria have described damage to staple meals and flour due to infestation by the red flour beetle. For example, a study in north-eastern Nigeria described the index of susceptibility of flour to infestation by this beetle as 19.65–20.76% in unpolished flour and 18.89–19.76% in polished flour [3], while another study from north-central Nigeria described the potential of this pest to destroy millet, wheat, cassava, yam, etc. [10,11]. Another recent study documented a lower mean weight loss of 8.75% for cassava and millet, on which the red flour beetle was grown compared with a higher weight loss seen in maize (25%) [12].

The control of this pest relies heavily on the use of chemical insecticides/pesticides [13] and several classes of insecticides are used, including pyrethroids, organophosphates, organochlorides, carbamates and abamectin [14,15]. However, the continuous application of pesticides in the field and granaries has accelerated the development of resistance in this agricultural pest to diverse insecticides from several classes [16], threatening the success of pesticide-based control measures [14,17]. For example, multiple resistance towards three different classes of insecticides (deltamethrin, abamectin and chlorpyrifos) has been reported in the Iranian population of the red flour beetle [13]. In contrast, susceptibility to pyrethroids (cypermethrin) was observed in the Egyptian population, which was resistant to pirimiphos-methyl [18]. One of the first studies on resistance in this beetle and its potential molecular mechanism, conducted over six decades ago, included a population from Nigeria which was resistant to malathion [17]. Unfortunately, since then, little effort has been invested to explore the resistance status of this pest in Nigeria and the molecular mechanisms of the resistance, despite the anecdotal reports of resistance to pesticides by farmers, granaries and flour mills. Most studies conducted in Nigeria evaluated the influence of cereal flour on the growth of this pest or the efficacy of traditional plants and/or their essential oils [19,20]. However, a recently published study on a population from Ondo, south-western Nigeria [21], has evaluated the impact of different rearing substrates on the insecticide resistance response in the red flour beetles, showing that composite (wheat + cassava) flour increases cypermethrin resistance.

Investigating the pesticide brands/formulations used for protecting stored grains and flours against the red flour beetle and the resistance status of this pest towards the active ingredients will provide information to enhance the rationale choice of the pesticides based on evidence, promoting food security in Nigeria and elsewhere. In this study, three populations of the red flour beetle from Kano, northern Nigeria, were investigated for pesticide resistance using four different insecticides (cypermethrin, dichlorvos, DDT and malathion) which constitute the major active ingredients used in the brand of pesticides in northern Nigeria to treat stored grains and flours. The beetles were identified morphologically and at the species level using molecular assays. The molecular mechanism of pyrethroid resistance was explored using cypermethrin, synergized with piperonyl butoxide, to detect the contribution of P450 monooxygenases. Amplification of fragments of the voltage-gated sodium channel encompassing the 929 and 1014 codons detected none of the two mutations, 929I and 1014F, associated with pyrethroid/DDT knockdown resistance, suggesting a limited role of target-site insensitivity towards these nerve agent pesticides.

## 2. Materials and Methods

### 2.1. Collection and Rearing of Beetles

The rust-red flour beetles were collected between April and May 2020, from three different locations within Kano Metropolis (11°58'17" N, 8°35'9" E): (i) Northern Nigeria Flour Mill company (NNFM), (ii) Yankaba Market and (iii) Rijiyar Zaki (R/Zaki) granary/flour mill. The parent stock of the beetles was recovered by sieving from infested flours and reared in an insectarium at Bayero University Kano under insectary conditions advised by the United States Department of Agriculture [22]. The insects were introduced to non-infested dried wheat flour, mixed with brewer's yeast at a ratio of 95:5 (*w/w*), and

maintained at 26 °C with photoperiods of 12 h:12 h light:dark. Emerged adults were periodically sub-cultured onto fresh flour/yeast mix to prevent overcrowding and starvation.

### 2.2. Morphological Identification of the Beetles

Morphological identifications of the eggs, larvae, pupae and adult beetles, as well as the sex of the adults and pupae, were performed microscopically, using a combination of the protocol provided by the Department of Entomology and Nematology, University of Florida, United States [7] and the work of Dia and colleagues [23]. Adult males were distinguished by swelling of the glands on their prothoracic legs. The sexes were confirmed by observing the genital papillae on the ventral side of the distal abdominal segment under a binocular microscope. The female pupae possess well-developed, protruding genital papillae, while the males possess reduced genital papillae compared to the females [24].

### 2.3. Insecticides Susceptibility Filter Paper Bioassays

The choice of insecticides to be tested was made based on the three most popular formulations/brands (Figure S1) used in storage facilities and warehouses in Kano to protect stored products from insect pests. These brands are: (i) DAKSH, made up of 100% *w/v* dichlorvos; (ii) Cypertrap, made up of 10% cypermethrin; and (iii) MULTIPHOS, made up of 560 g/kg aluminium phosphide plus inert material at 400 g/kg. However, phosphine was not tested due to its unavailability. Instead, in addition to dichlorvos and cypermethrin, DDT and malathion were also tested using the NNFM population. This is because previous studies have described populations of red flour beetles resistant to these two insecticides.

Analytical grade dichlorvos (98%), cypermethrin (100%), DDT (98%), and malathion (98%) were purchased from SIGMA Aldrich (Merck KGaA, Darmstadt, Germany). The bioassay was conducted using a modified protocol (Method No. 006) of the Insecticide Resistance Action Committee [25], with the beetles classified as fully susceptible where complete knockdown and mortality occurred or completely resistant when no knockdown/mortality was observed at 24 h. Nine different concentrations of cypermethrin, dichlorvos DDT, and malathion (ranging from 0.0625 to 10 mg/mL) were made from the stock solution of 200 mg/mL in Dow Corning (silicone) oil and acetone for cypermethrin and DDT, or olive oil and acetone for dichlorvos and malathion. Whatman filter papers were cut into 4 × 4 cm<sup>2</sup> and impregnated with 1 mL of each of the insecticide solutions above. The filter papers were dried in the dark at room temperature and kept in the fridge (4 °C) prior to use. The beetles were held without food for 1 h at 26 °C and 70% relative humidity prior to the tests.

For each concentration, four replicates of 20 F<sub>1</sub> beetles aged 2 to 4 days old were transferred into a 4 × 4 cm<sup>2</sup> plastic tray containing the 4 × 4 cm<sup>2</sup> insecticide-impregnated filter paper. The beetles were exposed for 5 h after which they were transferred into another 4 × 4 cm<sup>2</sup> plastic tray containing the 4 × 4 cm<sup>2</sup> non-impregnated paper and knockdown plus delayed mortalities were recorded at 24 h. Control groups were exposed to papers impregnated with acetone and carrier oil only.

### 2.4. Investigation of the Role of Metabolic Resistance Using Synergist Bioassay

To investigate the potential role of metabolic mechanism in resistance, synergist bioassays were conducted using adult beetles with piperonyl butoxide (PBO: an inhibitor of cytochrome P450 monooxygenase), adopting a previous protocol we have utilized for tomato leaf miner, *Tuta absoluta* [26]. In total, four replicates each of 20 F<sub>1</sub> beetles pre-exposed to 4% PBO-impregnated papers were transferred to filter papers impregnated with 1.5 mg/mL cypermethrin (a concentration that produced ~20% mortality) and maintained for 5 h, before being transferred into non-impregnated papers. Mortalities were recorded at 24 h. Two control groups were used: (i) comprise of four replicates of 20 beetles exposed to PBO-impregnated papers only and (ii) comprised of four replicates of 20 beetles exposed to acetone plus Dow Corning oil-impregnated papers.

### 2.5. Investigation of the Role of Target-Site Pyrethroid/DDT Insensitivity Resistance Mutations

To investigate the potential role of the target-site insensitivity resistance mechanism known to reduce the toxicity of pyrethroids and DDT, the presence of knockdown resistance (*kdr*) mutations in the voltage-gated sodium channel (VGSC) was investigated. The cDNA sequence of *T. castaneum* para sodium channel (XM962927.2) described in previous publications [27,28] was retrieved from GenBank and used to create the primers below, flanking the IIS4 to IIS6 transmembrane domain of subunit II of the VGSC, encompassing the 929th and 1014th codons, respectively. Nine beetles each, randomly selected from alive and dead following the exposure to cypermethrin, were used for the detection of the above mutations. Genomic DNA was extracted from the beetles using the ethanol precipitation of Livak [29]. The PCR mix comprises 2 µL of gDNA, 2.5 µL of 10× Taq A Buffer, ~0.4 µM (0.85 µL) each of forward (Tcast\_IIS4-IIS6\_vgscF2: 5'-CCGACTGTTGAGGGTTTTTA-3') and reverse (Tcast\_IIS4-IIS6\_vgscR2: 5'-GCTATTTTATTAGTGTCGTT-3') primers, 1.25 mM (1.5 µL) of MgCl<sub>2</sub>, 0.25 mM (0.2 µL) of dNTP mixes and 0.2 µL of KapaTaq DNA polymerase (KAPA Biosystems, Wilmington, MA, USA), in ddH<sub>2</sub>O. Amplification was carried out using the following conditions: initial denaturation of 3 min at 94 °C, followed by 35 cycles each of 30 s at 94 °C (denaturation), 60 s at 51 °C (primer annealing) and 30 s at 72 °C (extension). This was followed with 5 min final extension at 72 °C. The PCR amplicons were separated on an agarose gel stained with pEqGREEN and visualized using Ingenius 3 gel doc (Syngene, Cambridge, UK). Amplicons were purified using the Qiaquick purification kit (Qiagen, Hilden, Germany) and sequenced on both strands using the above primers, by GeneWiz [<https://www.genewiz.com/en-GB/>] (accessed on 12 December 2022).

### 2.6. Molecular Identification of the Beetles to Species Level

A total of 32 randomly selected F<sub>1</sub> beetles, 16 cypermethrin-alive and 16 dead, were used for DNA extraction. Genomic DNA (gDNA) was extracted [29] and fragments of the mitochondrial DNA *cytochrome oxidase* subunit I (COXI) were amplified to identify the beetles at the species level using a polymerase chain reaction [30]. The PCR reaction was carried out using the KAPA Taq DNA polymerase kit (KAPA Biosystems, MA, USA) in a total volume of 25 µL. The reaction mix comprises 2 µL of gDNA, 2.5 µL of 10× Taq A Buffer, ~0.4 µM (0.85 µL) each of forward and reverse primers, Tca33F26: 5'-GAATAGTAGGCACTTCATTAAGACTC-3' and Tca346R24: 5'-CCATGTGCAATGTTTGATGA GAGG-3', 1.25 mM (1.5 µL) of MgCl<sub>2</sub>, 0.25 mM (0.2 µL) of dNTP mixes and 0.2 µL of Taq DNA polymerase, in ddH<sub>2</sub>O. Amplification was carried out using the following conditions: initial denaturation of 3 min at 94 °C, followed by 35 cycles each of 30 s at 94 °C (denaturation), 60 s at 51 °C (primer annealing) and 30 s at 72 °C (extension). This was followed with 10 min final extension at 72 °C. PCR amplicons were separated using the agarose gel electrophoresis and visualized to confirm the presence of 337 bp fragments diagnostic of *T. castaneum*.

### 2.7. Data Analysis

Insecticide susceptibility bioassay data were corrected for mortality in the control using the Abbott's formula [31]. Linear-probit analysis of larval bioassay result for LC<sub>50</sub> was performed using PASW statistics 18 software: <http://www.spss.com.hk/statistics/> (accessed on 1 May 2022). Insecticide susceptibility bioassay results were analysed using Microsoft excel, which was also used to plot bar charts. A chi-square test of significance was performed to assess the significance of synergist bioassay using an online tool [<https://www.socscistatistics.com/tests/chisquare/default2.aspx>] (accessed on 1 May 2022)].

## 3. Results

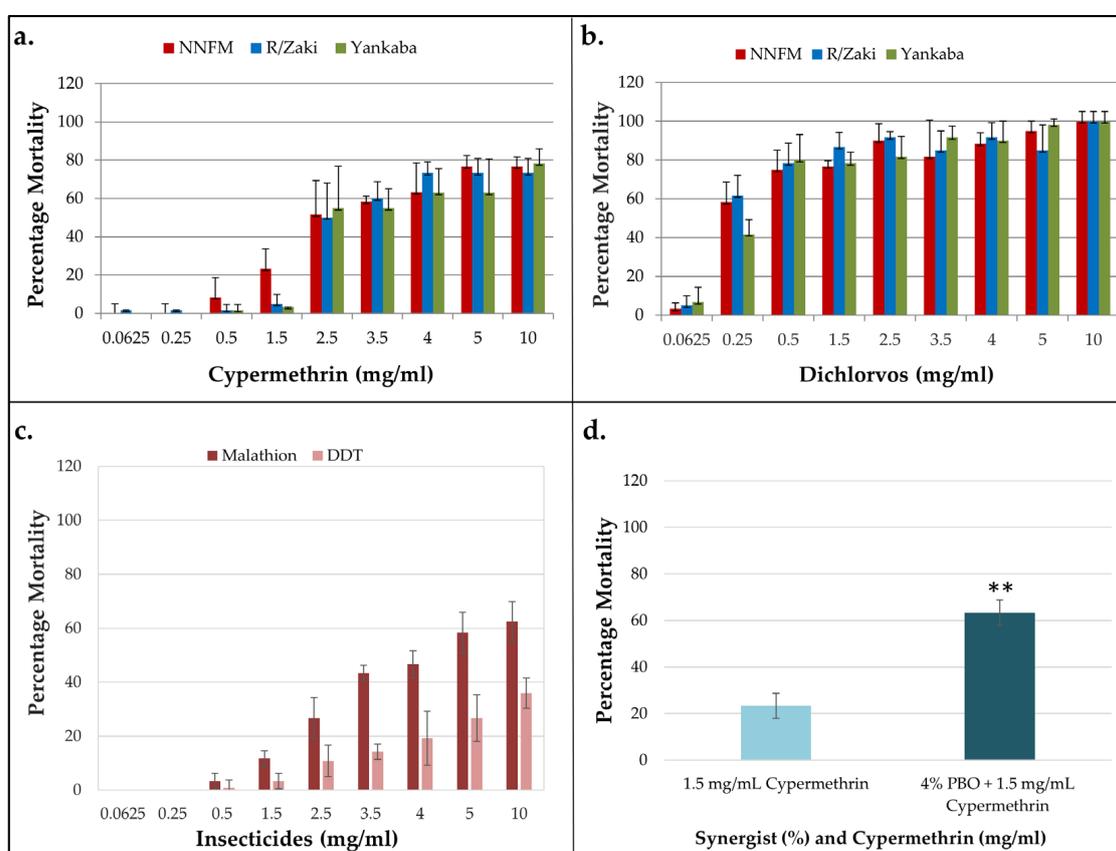
### 3.1. Morphological and Molecular Identification of Beetles to Species Level

The beetles were morphologically identified as *T. castaneum* (Herbst, 1797) based on morphological keys [7,23]. The bodies of adult beetles were flat, reddish-brown and measured about 3–4 mm long (Figure S2). The thorax is slightly darker than the elytra

and densely punctuated. The last three antennal segments were larger, almost square. The elytra have a conspicuous field of trichoid sensilla, with numerous gland openings when viewed from the dorsal side. Primers targeting the mitochondrial *cytochrome oxidase I* (*mtCOXI*) generated a band of 337 bp [30] for 31 samples, which were amplified successfully (Figure S3), confirming that the beetles were *T. castaneum*.

### 3.2. Insecticides Resistance Profile of the *T. castaneum* Population

Filter paper bioassays were conducted to assess the resistance profile of the *T. castaneum* beetles to four insecticides from three different classes. While samples from NNFM were tested with cypermethrin, dichlorvos, DDT and malathion, those from Yankaba and R/Zaki were tested with dichlorvos and cypermethrin only. Resistance was observed toward cypermethrin, for all concentrations, with no mortalities at 0.0625 mg/mL and 0.25 mg/mL for NNFM and Yankaba populations (Figure 1a).



**Figure 1.** Resistance profiles of rust-red flour beetles to various concentrations of insecticides. Each bar represents the percentage mortality from four individual replicates for each concentration of (a) cypermethrin, (b) dichlorvos, (c) malathion and DDT for the NNFM population only and (d) PBO synergist bioassay with cypermethrin. Error bars indicate the standard deviation of the means. \*\*  $p = 0.01$  for comparison between synergized and un-synergized bioassays.

Only around 50–55% of the flies were killed at the median concentration of 2.5 mg/mL and average mortalities of 76.7%, 73.3% and 63% were obtained from 5 mg/mL exposure for NNFM, R/Zaki and Yankaba beetles, respectively, which did not increase for the first two populations. The cypermethrin  $LC_{50}$ s (lethal concentrations that killed 50% of the beetles) for NNFM, R/Zaki and Yankaba beetles were 4.35, 4.45 and 5.46 mg/mL, respectively (Table 1).

**Table 1.** Result of probit analysis. Strength of cypermethrin, dichlorvos, malathion and DDT resistance in the three populations of *T. castaneum* studied.

Insecticide	Population	LC <sub>50</sub> (mg/mL)	95% CI	LC <sub>90</sub> (mg/m)	95% CI	Slope (SE)	χ <sup>2</sup>	df	p
Cypermethrin	NNFM	4.35	3.68–5.01	12.74	7.90–20.87	2.27 (0.18)	6.48	7	0.37
	R/Zaki	4.45	3.83–5.02	13.45	6.99–26.25	2.02 (0.14)	14.21	7	0.50
	Yankaba	5.46	4.64–6.27	12.81	7.35–20.06	1.76 (0.12)	41.45	7	0.40
Dichlorvos	NNFM	0.28	0.07–0.63	4.94	1.62–9.12	1.33 (0.08)	50.26	7	0.1
	R/Zaki	0.17	0.03–0.46	3.83	1.39–10.76	1.26 (0.28)	60.86	7	0.09
	Yankaba	0.35	0.06–0.63	2.67	1.59–5.71	1.47 (0.89)	36.22	7	0.15
Malathion	NNFM	3.71	1.72–5.92	15.41	11.46–23.85	2.313 (0.24)	3.56	7	0.73
DDT	NNFM	15.32	5.09–20.33	47.63	23.82–192.19	2.001 (0.35)	1.41	7	0.09

n (the number of adult *T. castaneum* used for each insecticide = 720); LC<sub>50</sub> = concentration that killed 50% of the experimental insects; CI = 95% confidence interval; SE = standard error, df = degree of freedom, p = p-value.

On the contrary, dichlorvos inflicted the highest mortality of all insecticides tested with even low concentrations of 0.25 mg/mL killing 58%, 60% and 40% of the NNFM, R/Zaki and Yankaba populations, while the median concentration of 2.5 mg/mL killed between 80% and 90% of the beetles (Figure 1b). LC<sub>50</sub>s of 0.28, 0.17 and 0.35 mg/mL were calculated, respectively, for NNFM, R/Zaki and Yankaba (Table 1).

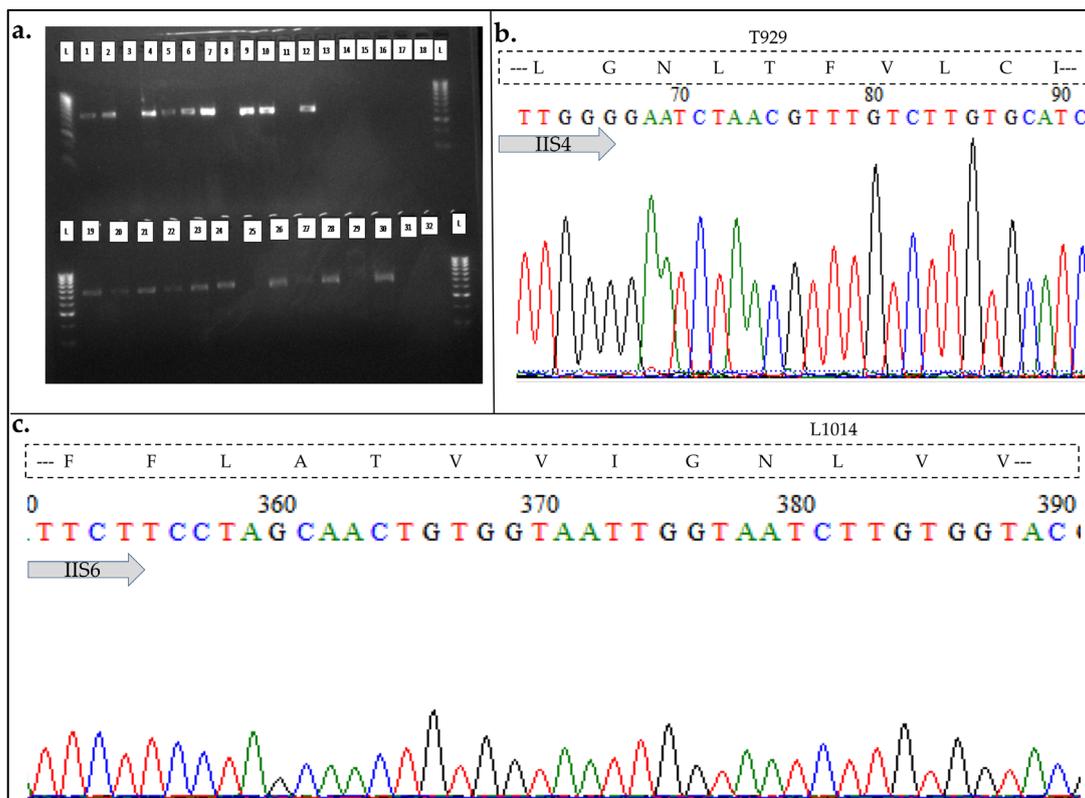
Resistance was observed towards malathion and DDT, with no mortalities at 0.0625 mg/mL and 0.25 mg/mL, and mortalities of 26.7% and 10.8%, respectively, at the median concentration of 2.5 mg/mL, which increased to only 58.3% and 26.7% at the 5 mg/mL (Figure 1c). The LC<sub>50</sub>s for malathion and DDT were 3.71 and 15.32 mg/mL, respectively.

### 3.3. Assessment of the Role of Metabolic Resistance in Cypermethrin Resistance

Synergist bioassay was conducted with PBO to investigate the role of CYP450 monooxygenases on cypermethrin resistance. Pre-exposure to 4% PBO for 1 h, followed by 1.5 mg/mL cypermethrin significantly restored susceptibility, with mortalities increasing approximately threefold, from 24.8% with 1.5 mg/mL cypermethrin alone to 63.3% in the synergised group ( $p = 0.01$ ) (Figure 1d).

### 3.4. Assessment of the Role of VGSC Target-Site Insensitivity *kdr* Mutations in Cypermethrin Resistance

A 550 bp fragment of the *T. castaneum* para sodium channel gene encompassing domains IIS4-IIS6 of the voltage-gated sodium channel was successfully amplified from the gDNA of nine each of cypermethrin-alive and cypermethrin-dead beetles, and sequenced (Figure 2a) to investigate the presence of T929I and L1014F *kdr* mutations. For both alive and dead beetles, the above mutations were absent, with the codons ACG (Figure 2b) and CTT (Figure 2c) coding for threonine and leucine, respectively, present in both the two contrasting phenotypes.



**Figure 2.** Amplification and analysis of fragments of voltage-gated sodium channel gene. (a) Amplified segments of the *T. castaneum* VGSC. L is hyper-ladder IV DNA ladder (Bioline 100–1013 bp (40–200 ng/band)); (b) and (c) Analysis of the polymorphism of fragments of the VGSC gene spanning the T929I and L1014F *kdr* mutations codons, respectively.

#### 4. Discussion

Reducing post-harvest and storage loss of food stocks is of crucial importance to promoting food availability and security in sub-Saharan Africa, where a significant food deficit is experienced in most countries [32], partly due to biotic agents like agricultural insect pests that contaminate and destroy food items. However, one of the major challenges in the control of pests is resistance in most insect species. In this study, we established resistance to three chemicals which are used as pesticides for control of the rust-red flour beetle affecting grains and stored flour in northern Nigeria and explored the possible molecular mechanism of pyrethroids (one of the major ingredients used in pesticide formulations) resistance.

Morphological and molecular identification of the beetles confirmed that they are *T. castaneum* (Herbst, 1797). Several studies have utilized a combination of morphological and molecular approaches to differentiate the species of beetles infesting stored food produce [5,23,30,33] and its ability to destroy the grains, cereals and flour in Nigeria, Africa and globally [7,18,34,35]. Indeed, many studies have identified the red flour beetle as an important pest that destroyed flour in Nigeria. For example, Ehisianya and colleagues [12] have demonstrated the role of this beetle in the invasion and destruction of different types of flour including maize, wheat, millet, sorghum and cassava flour from Umudike, southern-eastern Nigeria. Another study on the major flour pests from south-western Nigeria has revealed this beetle as the major species alongside *Tenebrioides mauritanicus* and *Dinoderus porcellus* [36]. A study from north-eastern Nigeria [4] has also described this pest to be predominant among flour pests in Maiduguri.

The development and spread of resistance in primary and secondary agricultural pests are of interest to farmers and warehousing businesses [37]. Furthermore, without understanding the resistance profile of the major pests and their mechanism, it will not be

possible to control the pest through resistance management. In this study, cypermethrin resistance was observed in all three populations of *T. castaneum* studied. A recent paper on a population from Ondo, south-western Nigeria [21] has evaluated the impact of different rearing substrates on the insecticide resistance response of red flour beetles, showing that composite (wheat + cassava) flour increases cypermethrin resistance. Several studies have reported similar or contrasting resistance cypermethrin phenotypes. For example, in Egypt, a recent study [38] has revealed that cypermethrin exhibited low toxicity to a population of *T. castaneum* when tested using the thin-film technique, with an LC<sub>50</sub> value of 2.20 mg/mL obtained 24 h after treatment, a value which is on average half the LC<sub>50</sub>s from our study. On the contrary, Naeem and colleagues [39] had described a population of *T. castaneum* highly susceptible to cypermethrin, with mortalities of >94% when exposed to 5 mg/mL of cypermethrin for 24 h. The LC<sub>50</sub> obtained by Naeem and colleagues (0.09%, ~0.9 mg/mL) was four to five times lower than LC<sub>50</sub>s obtained in all three populations from Kano. Another study conducted by Khalequzzaman and Khanom [40] reported very high toxicity of cypermethrin in adult flour beetles from Bangladesh, which is contrary to our findings. High resistance of *T. castaneum* to type II pyrethroid, cypermethrin had also been previously reported from the Italian population [35].

The highest susceptibility was obtained from dichlorvos, even at low concentrations, which is similar to the findings of Andric et al. [41], who reported high mortalities (>95%), and another study which reported absolute mortality upon exposure of Indian populations to this organophosphate [42].

Unfortunately, only the NNFM population was screened for malathion and DDT resistance due to the unavailability of these insecticides to bioassay all three populations. The finding of high malathion resistance in our study was not surprising. Malathion resistance was described several decades ago in the Nigerian population of the red flour beetle, and its potential molecular mechanism was explored [17,43]. Several other studies have described malathion resistance in *T. castaneum*, for example, studies from Australia [44,45].

The highest resistance was observed with DDT, which was not surprising, as one of the pioneering studies on resistance in the red flour beetle was a selection study in which a colony with a slight tolerance at 1.8 ppm was selected for several generations until stocks with a resistance factor of  $\times 166$  were generated [46].

PBO is increasingly being used as a synergist to investigate the role of P450 monooxygenases in metabolic resistance [47], for example in several studies targeting insect pests of public health and agricultural importance, using mostly pyrethroids [26,48–50]. The contribution of monooxygenases in *T. castaneum* resistance has been demonstrated in insects by [51] and other researchers. For example, using a combination of functional genomics and reverse genetics approaches, Zhu and colleagues [50] have shown that overexpression of a P450 CYP6BQ9 was associated with deltamethrin resistance in the QTC279 strain of *T. castaneum* compared to the susceptible strain. In a similar finding, an ATP-binding cassette transporter, TcABCG-H has been found overexpressed in response to insecticide induction [52]. The synergist assay conducted in our study demonstrated that P450 monooxygenases-mediated metabolic mechanism is associated with cypermethrin resistance.

The study of knockdown resistance (*kdr*) mutation in the VGSC has led to the identification of several mutations that alone or in combination with other mechanisms were known to confer resistance to pyrethroids in arthropod species [53]. Further, previous studies have associated polymorphisms in the *T. castaneum* voltage-gated sodium channel (VGSC) with insecticide resistance [27,52–55]. The absence of the 929I and 1014F *kdr* mutations in the VGSC from the *T. castaneum* in this study suggests the preeminent role of the metabolic mechanism in pyrethroid resistance. The T929I mutation is associated with DDT resistance in the maize weevil, *Sitophilus zeamais*, in a previous study [28]. Resistance in pyrethroid-resistant rice weevils was associated with the combination of the L1014F *kdr* mutation, and increases in walking and detoxification activities, while the T929I mutation combined with

increases in walking activity were the primary pyrethroid resistance mechanisms in maize weevils [27].

## 5. Conclusions

In conclusion, the rust-red flour beetle, *T. castaneum* from Kano, northern Nigeria is multiple resistant to several pesticides in use for its control (including pyrethroids, DDT and organophosphates), threatening the quality and quantity of stored grains, cereals and flour. The resistance is probably driven by metabolic mechanisms, potentially the cytochrome P450 monooxygenases. The absence of the two major *kdr* mutations 929I and 1014F suggests the lesser role of target-site mutations in pyrethroids and/or DDT resistance. These findings highlight the challenges associated with the control of this secondary pest of stored products and underscore the need to explore alternative approaches for its control, as well as using synergists against the major metabolic genes driving the resistance, such as the CYP450s.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agrochemicals2020012/s1>, Figure S1: Three of the most widely used pesticide formulations for control of beetles of stored flour in northern Nigeria., Figure S2: Agarose gel of amplified fragments of mitochondrial *cytochrome oxidase I* for identification of the red-rust flower beetles from Kano, Nigeria, to species level. The first and last lanes (L) of each panel is a hyper ladder IV DNA ladder (Bioline 100–1013 bp (40–200 ng/band)). 31 samples produced 337 bp band sizes characteristic of *T. castaneum*., Figure S3: Morphology of the posterior and anterior body of red flour beetles from Kano. Pictures were captured using a stereomicroscope at  $\times 2$  magnification. Files S1: Genotyping of *kdr* mutations in cypermethrin-alive and -dead *T. castaneum*. Sequences of the fragment of the voltage-gated sodium channel encompassing the IIS4-IIS6 transmembrane domain, harbouring the T929I and L1014F mutations.

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