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Special Issue Reprint

Advances in Biomimetics

Patents from Nature

Edited by

Stanislav N. Gorb, Longjian Xue, Barbara Mazzolai and Phillip B. Messersmith

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Guest Editors

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About the Editors

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Advances in Biomimetics: Patents from Nature

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Biomimetics seeks to translate principles from living systems into innovative engineering solutions by drawing on the remarkable efficiency, adaptability, and multifunctionality found in nature. Biological materials, structures, and processes are inherently hierarchical, combining mechanisms across nano-, micro-, meso-, and macroscales to achieve optimized performance under complex environmental conditions. Understanding these multiscale interactions is essential for identifying fundamental design rules and transferring them into technical applications. This Special Issue highlights recent progress in key areas of biomimetics, including materials and structures, surfaces and interfaces, locomotion and control, and biomedical systems, sometimes integrating and bridging these domains.

The contributions collected in this issue reflect a strong interdisciplinary effort, combining theoretical, experimental, and computational approaches from biology, physics, materials science, and engineering. They not only deepen our understanding of natural systems—from adhesive mechanisms and fluid transport to locomotion strategies and structural design—but also demonstrate how these insights can be translated into functional technologies. Particular emphasis is placed on the identification of underlying biological principles and their application in the development of advanced materials, surfaces, and adaptive devices. By bringing together diverse perspectives and methodologies, this Special Issue fosters the transfer of knowledge between disciplines and supports the continued advancement of biomimetic research toward practical and scalable innovations.

Among the contributions to this Special Issue, the review by Guidi et al. [1] addresses the pressing challenge of valvular heart disease, which continues to represent a major global health burden. Current prosthetic heart valves, although widely used, still fail to replicate the adaptive, regenerative, and long-term functional properties of native valves. Tissue-engineered heart valves (TEHVs) have therefore emerged as a promising alternative, representing living replacements capable of growth, remodeling, and physiological integration. However, despite considerable progress, their clinical translation remains limited, highlighting the need for design strategies that move beyond simple material substitution toward the development of functionally mature constructs. Adopting a biomimetic perspective, the authors emphasize native heart valves as a model system characterized by hierarchical organization, anisotropic mechanical behavior, mechanoresponsive cellular activity, and tightly regulated remodeling processes. The review integrates current knowledge in valve biology and mechanobiology with advances in scaffold design, bio-functionalization, biomechanical conditioning, and emerging fabrication technologies.

Particular attention is given to how biomimetic approaches can reproduce extracellular matrix architecture, regulate immune and thrombotic responses, and guide tissue maturation through controlled mechanical stimulation. The authors conclude that successful clinical translation will require a coordinated design strategy that combines structural anisotropy, immune modulation, and dynamic conditioning, thereby illustrating how biomimetics can provide a unifying framework for the development of next-generation regenerative medical devices.

Biological adhesion and anti-adhesion represent two complementary strategies that are central to many natural systems and increasingly relevant for biomimetic applications. On the one hand, numerous organisms, such as insects, spiders, and geckos, have evolved highly efficient attachment mechanisms, while, on the other hand, plants have developed strategies to prevent adhesion or even actively trap animals. The contribution by Becker et al. [2] in this Special Issue investigates the mechanical properties of adhesive pad fluids in insects, which play a crucial role in mediating attachment. Using atomic force microscopy-based nanoindentation, the authors reveal that these fluids exhibit distinct viscoelastic behaviors, ranging from nearly inviscid to highly viscous or even rigid droplet types. Such variability suggests a non-uniform composition and indicates that insects may deploy functionally different fluid states to adapt adhesion to changing conditions. These findings advance our understanding of dynamic, fluid-mediated adhesion and provide inspiration for the development of soft, tunable, and reversible adhesive systems in engineering.

In contrast, anti-adhesive strategies are exemplified by carnivorous plants, such as pitcher plants, which exploit surface properties and fluid characteristics to capture prey. The study in this issue by Gorb et al. [3] demonstrates how different components of the pitcher trap syndrome—including trap geometry, inner surface coatings, and fluid properties—contribute to trapping efficiency. Biomechanical experiments using biomimetic artificial pitchers show that surfactant-containing fluids with strong wetting properties are particularly effective in immobilizing insects. In addition, microstructured coatings mimicking plant particular epicuticular waxes reduce contact between insect feet and surfaces and promote contamination of their adhesive organs, thereby impairing attachment. Together, these studies highlight the interplay between adhesion and anti-adhesion in biological systems and illustrate how both principles can be translated into innovative technologies, ranging from advanced gripping devices to anti-fouling and trapping surfaces.

The contribution by Varnier et al. [4] addresses fluid transport in biological and bioinspired systems, highlighting how directional control of flow emerges from structural design rather than active pumping alone. The authors investigate hydraulic asymmetries in tubular channels as a fundamental mechanism underlying biological valves, physiological transport systems, and engineered passive devices, such as Tesla valves. Using the concept of diodicity—defined as the ratio of pressure drops required to drive identical flow rates in opposite directions—the study systematically analyzes how geometry and internal structures generate directional bias in fluid transport. Through numerical simulations in both two- and three-dimensional configurations, the authors demonstrate that channel geometry alone establishes a baseline level of hydraulic asymmetry. This effect is further modulated by the presence of internal obstacles, which may be rigid or deformable, enabling either continuously open channels or valve-like structures capable of complete flow blockage. The extension to three-dimensional systems reveals that increased dimensionality and structural adaptability significantly enhance diodicity, amplifying directional control. These findings provide a unifying framework for understanding passive flow regulation in biological systems and offer valuable design principles for the development of efficient, low-energy biomimetic valves and microfluidic transport devices.

The contribution by Yang et al. [5] explores bioinspired robotic locomotion in aquatic environments, focusing on the integration of surface structure and interfacial fluid phenomena for controlled propulsion. The authors investigate shark skin as a multifunctional interface, combining a hierarchical arrangement of three-dimensional dermal denticles with an underlying structural layer that is known to contribute to passive drag reduction. Beyond this well-established function, the study addresses the less explored possibility of active drag modulation through surface-mediated fluid effects. To this end, the authors develop a 3D-printed swimming robot equipped with a porous substrate and biomimetic denticle structures, enabling a controlled release of surfactants that generate Marangoni-driven propulsion. By tuning denticle geometry and spatial arrangement, the system achieves programmable swimming trajectories and on-demand motion control. Additionally, the incorporation of superhydrophobic surface treatments reduces hydrodynamic resistance and further enhances propulsion efficiency. The experimental results show that denticle configurations with a 30° attack angle provide optimal performance under both hydrodynamic and aerodynamic conditions. These findings suggest that mucus secretion on shark skin may actively contribute to drag reduction via Marangoni effects, offering new principles for the design of autonomous, highly efficient aquatic robots.

This Special Issue also addresses bioinspired locomotion control in legged robotic systems, focusing on smooth and adaptive transitions between different gait patterns. Rostro-Gonzalez et al. [6] propose a control framework based on spiking neural networks functioning as central pattern generators (CPGs), which produce coordinated spike trains that emulate neural activity observed in biological systems. These signals are translated into synchronized motor commands, enabling a hexapod robot to execute distinct locomotion patterns, including walking, jogging, and running. A key aspect of the approach is the use of a similarity metric (SPIKE-synchronization) to identify optimal transition points between gaits, ensuring smooth switching without disrupting stability. This mechanism is complemented by force-sensing resistors embedded in the robot's legs, which allow the real-time assessment of terrain stiffness and adaptive selection of the most appropriate locomotion pattern. Experimental validation on a physical hexapod robot across different surface conditions demonstrates improved balance, reduced abrupt actuator changes, and enhanced energy efficiency. Overall, the study [6] highlights how biologically inspired neural control strategies can significantly improve adaptability and robustness in legged robotic locomotion systems.

The authors included in this Special Issue also explore the use of biological neural network architecture for computational intelligence, demonstrating how the connectome of *Drosophila melanogaster* can be exploited as a reservoir for time-series prediction tasks. Costi et al. [7] utilize recently available full connectome data to construct an Echo State Network, directly mapping the biological connectivity matrix into a computational framework. By selecting subsets of highly connected neurons and applying different selection strategies that either preserve or modify the proportional representation of neuron classes, the authors derive computationally efficient reservoir architectures inspired by the fruit fly brain. The performance of these biologically derived reservoirs is evaluated against standard state-of-the-art implementations. The results show that connectome-based architectures exhibit significantly improved robustness to overfitting, particularly in regimes where conventional models tend to fail. Further analysis using hybrid configurations—combining biological topology with random synaptic weights and vice versa—demonstrates that both network structure and weight distribution contribute to the observed performance gains. Even when the full connectome is used as a reservoir, the system maintains strong generalization ability, achieving low prediction errors with reduced regularization. These findings highlight the

potential of biological neural architectures as a powerful blueprint for developing more robust and efficient computational models in machine learning and artificial intelligence.

Another contribution in this Special Issue focuses on control strategies for bioinspired soft actuators in rehabilitation robotics, particularly pneumatic artificial muscles (PAMs), which are widely used due to their lightweight structure and inherent compliance. Brown and Xie [8] address the major challenge of accurately modeling and controlling the strongly nonlinear dynamics of PAM systems. To overcome these limitations, the authors develop a model predictive control (MPC) framework based on system models optimized using particle swarm optimization (PSO), enabling improved prediction and real-time motion control performance. The proposed MPC approach is systematically compared with classical PID control, PSO-tuned PID control, and iterative learning control (ILC) under a range of dynamic conditions, including varying setpoints, external loads, and disturbances. Experimental results show that conventional PID control exhibits the largest tracking errors and undesirable oscillations, while PSO-PID improves performance but still lacks stability in human–robot interaction scenarios. ILC achieves good convergence and disturbance rejection but struggles with varying input frequencies. In contrast, the MPC approach consistently demonstrates superior performance, achieving the lowest tracking errors, fast response to changing setpoints, and stable operation without requiring a learning phase. These results highlight the advantages of predictive, model-based control strategies for enhancing the safety, precision, and adaptability of soft robotic systems in biomedical applications.

A further contribution in this Special Issue highlights advances in bioinspired optics and functional nanostructured surfaces through the study of naturally occurring corneal architectures in arthropods. Kryuchkov et al. [9] address the challenge of producing efficient, cost-effective, and biocompatible nanopatterned coatings, which are essential for applications in cell signaling, drug delivery, and optical surface engineering. As a biological model system, beetles (Coleoptera) are investigated due to their highly diverse and adaptive corneal nanostructures, which serve both optical and protective functions. Using atomic force microscopy, the authors identify dynamic dimpled nanocoatings capable of transforming into maze-like or nipple-like surface morphologies. These transitions are interpreted as a result of reversible, self-assembled processes driven by surface adhesion effects. A key molecular component of this system, cuticular protein 7 (CP7), is identified and shown to possess intrinsic self-assembly properties that enable controlled nanopattern formation *in vitro*. By replicating these mechanisms, the study demonstrates a route toward tunable and reversible bioinspired nanocoatings. These findings not only provide insight into the evolutionary optimization of optical nanostructures in insects but also open new perspectives for the development of adaptive, bioinspired optical materials and surface technologies.

The final contribution in this Special Issue addresses bioinspired protective systems, focusing on how natural armors achieve an effective balance between mechanical protection and flexibility. Pantano and Baiamonte [10] investigate scale-like structures inspired by the segmented armor of fish and armadillos, where geometry and hierarchical organization play a crucial role in mechanical performance. In contrast to conventional flat artificial scales, the proposed designs feature three-dimensional architectures with a vertical support anchored to the substrate and an inclined protective surface, enabling a more realistic replication of biological armor functionality. Two distinct configurations—overlapping and staggered scales inspired by elasmoid fish structures—are analyzed using finite element simulations to evaluate puncture resistance and deformability. The results demonstrate that subtle geometric modifications can lead to pronounced differences in mechanical behavior: one configuration maximizes resistance to penetration, while the other enhances

flexibility and compliance. This trade-off reflects a key principle in biological protective systems, where performance is governed by structural tuning rather than material changes alone. The study not only deepens the understanding of natural armor mechanics but also provides practical design guidelines for advanced protective equipment, including lightweight body armor, gloves, and thermal protection systems.

This Special Issue of *Biomimetics*, enriched by high-quality theoretical, experimental, and review contributions from researchers in biology, physics, materials science, and engineering, will undoubtedly find a broad and diverse readership among scientists engaged in this rapidly expanding field. The collected works not only demonstrate the depth and diversity of current biomimetic research but also highlight its strong interdisciplinary nature and growing technological relevance. By bridging fundamental biological insights with engineering applications—from adhesion and anti-adhesion systems to fluid transport, locomotion, nanostructured optics, and protective materials—this issue underscores the transformative potential of biomimetics as a unifying framework for innovation. It is expected to serve as a valuable reference for researchers, engineers, and designers seeking to translate principles from nature into next-generation technologies.

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Article

Effect of Different Characters of the Pitcher Trap Syndrome in *Nepenthes* on Insect Trapping Efficiency: A Biomimetic Approach

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Abstract

The aim of our study was to determine the importance of different pitcher syndrome characters (size of the trap, the presence of inner microscopic surface coverage, physical properties of the pitcher fluid) for insect trapping efficiency using artificial, “biomimetic” pitchers. We performed trapping experiments with *Drosophila melanogaster* flies, applied cryo scanning electron microscopy for characterization of the topography of surface coatings and visualization of their contaminability effects on insect attachment organs, and conducted contact angle measurements with different liquids used in experiments. The type of the liquid used as the pitcher fluid had the most important impact on the trapping efficiency; surfactant-containing liquids exhibiting strong wetting properties provided a high number of trapped flies. The diameter of the trap rather than its height influenced insect trapping efficiency; apparently, because wider traps provide a larger space for more insects to get into a trap, they captured more flies in comparison to narrower traps. The presence of both the calcium carbonate and kaolin coatings mimicking the epicuticular wax coverage inside pitchers in many *Nepenthes* species additionally contributed to the trapping success due to a reduction of contact between insect feet and the trap surface and to contamination of flies’ attachment organs by detached microparticles.

Keywords: adhesive pad; carnivorous plant; claw; coating; contact angle; digestive fluid; *Drosophila melanogaster*; epicuticular wax; microparticles; *Nepenthes alata*; surfactant

1. Introduction

Carnivorous plants, usually called insectivorous plants, are distributed worldwide and comprise more than 860 species from 18 genera of angiosperms growing commonly in nutrient-poor habitats [1–3]. In addition to photosynthesis, these plants rely on capturing small animals, typically arthropods (mostly insects), and possess a cluster of characters and mechanisms (traits) that altogether build up the carnivorous syndrome, ensuring the following six main functions: (1) to attract, (2) retain, (3) trap, (4) kill animals, (5) digest the captured prey, and (6) absorb the derived nutrients [4,5]. All of this is achieved by the use of specialized trapping organs (in most cases, transformed and specialized leaves)—either passive ones that do not employ movement (pitfall, lobster pot, passive flypaper) or active motile ones that apply various movements to catch prey (active fly paper, snap or steel trap, mousetrap) [4,5]. Newly, a passive motile trapping mechanism, which is based on a rain-actuated ‘springboard’ movement of one part of the passive trap, was discovered [6–8].

Among passive pitfall traps, which are gravity-driven and characteristic of *Nepenthaceae*, *Sarracenaceae*, *Cephalotaceae*, and some *Bromeliaceae*, pitchers of tropical

pitcher plants *Nepenthes* L. (nearly 200 species according to [9]) (Nepenthaceae) are classical examples of such trapping organs. Most representatives of this Palaeotropic genus are vines or subscandent shrubs, typically growing terrestrially or in a few cases epiphytically [10]. All species produce pitchers, usually of two types (i.e., are dimorphic): ovoid/globose terrestrial (lower) ones bearing wing-like structures, which contact the ground, and funnel-shaped/cylindrical aerial (upper) ones lacking “wings” [10,11]. Whereas lower pitchers are developed by young immature plants, upper ones appear when plants reach a certain age [12]. Interestingly, it has been found that in some *Nepenthes* species, different types of pitchers are specialized in capturing different prey taxa [13–17].

Developed at the tips of the tendrils coming from the tips of the leaf blades (Figure 1A), *Nepenthes* pitchers have a zoned structure on the inner surfaces and can be divided into several regions (zones), which differ in both morphology and presence of specialized microstructures and are related to different functions (Figure 1B,C) [4,18]. The lid and peristome, a collar-shaped structure around the pitcher mouth, play the main roles in prey attraction primarily due to numerous extrafloral nectaries (Figure 1B,C) and comprise the so called “attractive zone” (according to [4]), although also their coloration pattern [4] and the presence of the peculiar tomentose tissue near the peristome [19,20] can contribute to the attraction of prey animals in particular *Nepenthes* species. Additionally, the lid not only covers the pitcher mouth and offers shelter from heavy rain [21,22] but may also assist in prey capture due to the downward orientation, which affects the attachment negatively [23]. Presently, it was found that rain drops can actuate the ‘springboard’ movement of the lid in *N. gracilis* Korth. and *N. pervellei* Blume that catapults insects into the trap [6–8,24]. As for the peristome, recent experimental studies have revealed its key role in prey capture. Due to combination of the hydrophilic surface chemistry, overlapping and anisotropic arrangement of epidermal cells, specific surface roughness, and the presence of hygroscopic nectar, the peristome achieves extremely high wettability and slipperiness, which cause insects to slip by hydroplaning on a thin liquid film and to fall down inside the pitcher [22,23,25].

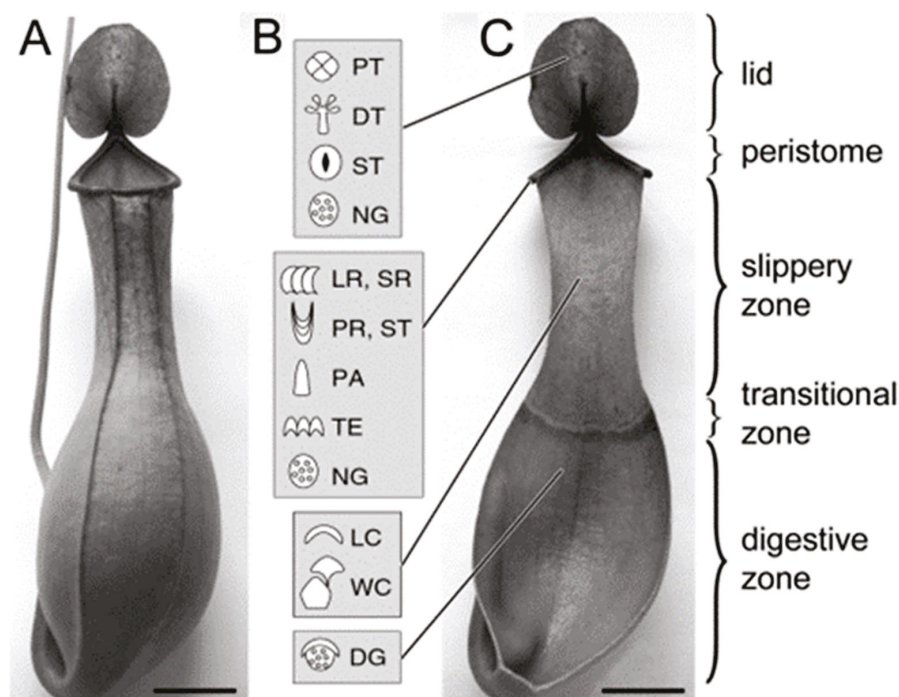


Figure 1. *Nepenthes alata* Blanco pitcher: general view (A); dissected pitcher with functional zones (C); schematic representation of surface structures characteristic for each zone (B). Scale bars: 2 mm. DG,

digestive glands with hoods; DT, dendroid trichomes; LC, lunate cells; LR, large ridges of the first order; NG, extrafloral nectar glands; PA, papillae; PR, parabolic ledges; PT, peltate trichomes; SR, small ridges of the second order; ST, stomata with guard cells; TE, teeth of the inner arm margin; WC, wax projections. Adapted from [26].

The second zone previously called a “conductive, slippery zone” [4,18,27,28] is characterized by the presence of lunate cells (modified stomatal guard cells) and epicuticular wax coverage on the inner pitcher wall (Figure 1B,C). It is often referred to as a “wax-coated”, “wax secreting”, or “waxy” zone (e.g., [4,21,29]). The lunate cells are responsible for surface anisotropy; insects easily move downward, in the direction of the pitcher bottom, but cannot interlock with their claws to concave, down-oriented hoods of lunate cells and, therefore, are not able to climb upward and escape from the pitcher [4,30–32]. The wax coverage, which is composed of one or more superimposed layers of wax platelets protruding perpendicular from the pitcher wall (e.g., [26,28,33,34]), reduces insect attachment primarily due to the presence of critical surface microroughness created by wax particles [33,35] and contamination of insect attachment organs by detached particles [21,29,30,32,33,36]. It has been also suggested that since the wax coverage exhibits highly hydrophobic properties and low free surface energy [26,30,37,38], it minimizes insect attachment due to very low capillary forces. Previously, this pitcher zone was considered to play the main role in prey capture (e.g., [4,21,30]), whereas recently, it is believed to be associated principally with the retention of prey and only secondly—with the initial trapping. However, comparative morphological studies of two pitcher zones primarily relevant to prey catching (the peristome and slippery zone) found (1) different types of slippery zones (with well-developed two-layered wax coverage, with greatly reduced wax coverage composed of solitary wax protrusions, and without wax) and (2) significant negative correlation between the length of the slippery zone and the width of the peristome [34,39]. Based on the analysis of the relationship between pitcher macromorphology and microstructure of the slippery zone, two types of *Nepenthes* pitchers have been proposed according to the main trapping mechanism: (1) based predominantly on the slippery waxy zone and (2) based on the peristome.

The next zone lacking any special surface structures is attributed as a “transitional zone” between the waxy and digestive zones (Figure 1C) [34] and is functionless according to [4].

The deepest zone inside *Nepenthes* pitchers, which bears numerous digestive glands, is called “digestive” or “absorptive” (Figure 1B,C) [4]. It is mainly associated with prey utilization—secretion of digestive enzymes and other molecules related to prey digestion, absorption, and transport of gained nutrients (e.g., [4,40–45]). Although not serving as a specialized trapping zone, the digestive zone can hinder the escape of insects from the pitcher; downward-directed epidermal lips that partially shield digestive glands do not provide a foothold for insect claws similar to the lunate cells of the slippery zone [4,18], and the secretion covering the surface inhibits attachment of insect pads [4]. Later experimental studies on *N. alata* and *N. × ventrata* Hort. ex Fleming revealed the contribution of this zone to the retaining function of the pitchers [30,46]. Firstly, frictional anisotropy of the glandular zone in terms of its influence on insect attachment was proven, and secondly, the adhesive (“gluing”) effect of the glandular secretion, at least at adhesive areas on the surface, impeding the locomotion of some insects was demonstrated.

The digestive fluid inside the pitcher, besides its important role in prey digestion, serves to coat, suffocate, and quickly drown animals downfallen onto the fluid surface, which is surface active [4]. Moreover, according to several recent studies, the pitcher fluid in some *Nepenthes* species exhibits certain viscoelastic properties that prevent insects from pulling themselves out of the fluid [15,47,48]. It has been shown for *N. rafflesiana*

Jack that the pitcher fluid viscoelasticity plays a more important role in prey retention compared to the slippery zone (both lunate cells and epicuticular waxes) [15,47]. It has also been suggested that the digestive fluid for prey capture (retention) in *Nepenthes* species lacking a slippery zone or slippery wettable peristome is fundamentally important [15,47]. Interestingly, based on the investigation of 23 *Nepenthes* species, Bonhomme et al. [48] found an inverse relationship between the viscoelasticity of the digestive fluid and the amount of wax produced in pitchers, suggesting the predominance of one of these two trapping mechanisms depending on the plant species, growing conditions, and type of prey. Recently, it has been reported that the high acidity of the digestive fluid can also contribute to the pitcher's trapping success [17,49].

Thus, there is a great amount of older and recent literature suggesting, examining, and proving the roles of different characters of the carnivorous syndrome in prey capture by *Nepenthes* pitchers, which is presented above, as well as a number of rather new publications in main or partly reviewing different aspects of trapping mechanisms in these pitchers (e.g., [8,10,12,17,45,50–52]). In particular, it has been found that the use of various trapping mechanisms (peristome-based, wax-based, or viscoelastic-fluid-based) is driven by climate: *Nepenthes* species with peristome-based and viscoelastic fluid-based mechanisms are associated with humid conditions, where the viscoelastic-group of species has the most restricted area, whereas the wax-based mechanism is employed in both humid and more seasonal regions [51]. The comparative study of the pitcher characters and prey composition in seven *Nepenthes* taxa from the same natural habitat has demonstrated that generalist carnivorous species had a sweet odor, wide pitcher opening, and acidic pitcher fluid, whereas different combinations of morpho-functional traits (capture syndromes) were developed for prey specializations, such as (a) extrafloral nectar production, fluid acidity, and slippery waxy walls for ant trapping, (b) narrow cylindrical pitchers, rim of edible trichomes or symbiotic association with ants, and non-viscous fluid for termite capture, and (c) odor, funnel-shaped pitchers, large pitcher opening, and acidic viscoelastic fluid for flying insects [17]. Later, based on functional traits present in their pitchers, *Nepenthes* species have been classified into 12 different functional types, with a fundamental division between wax-bearing and wax-lacking species [12]. As an ancestral type, ovoid/cylindric pitchers with an apical, horizontal opening, clearly separated a basal, glossy, fluid-containing, digestive part and an upper matt, whitish or greyish part covered by microscopic wax structures have been proposed. A recent study on the springboard trapping mechanism [6,7,24] found in two *Nepenthes* species that requires three independent components, such as horizontal orientation of the lid, slippery wax-bearing lower surface, and pivoting oscillations, showed that this new composite mechanism evolved not through directional selection in the component traits but arose convergently by “spontaneous coincidence” of a new beneficial trait combination [8].

The aim of our study was to determine the importance of different pitcher syndrome characters, such as the size of the trap, the presence of inner microscopic surface coverage, and physical (wetting) properties of the pitcher fluid, for pitcher trapping success. For simplicity, we did not consider here either the pitcher peristome or the surface anisotropy characteristic for either the slippery waxy or glandular zone; their impacts on prey trapping and retention have been experimentally shown previously [23,25,30–32,46].

Previous experimental studies on the functional significance of different pitcher zones and their features in relation to prey capture were performed using native trapping organs, often with live plants, which limits the ability to systematically manipulate individual traits and assess their independent functional significance. In our study, for the first time, we applied artificial, “biomimetic” pitchers such as plastic tubes (lacking both the peristome and anisotropy in the inner pitcher surface), where we imitated different functionally

significant pitcher characters. This approach made it possible to experimentally vary certain parameters in order to examine their importance for prey trapping. Such controlled biomimetic approaches that could disentangle the interactions between pitcher morphology, surface microstructure, and fluid properties are largely lacking, preventing a mechanistic understanding of how each feature contributes to prey capture and retention. A similar methodology of mimicking biological structures for a deeper understanding of functional principles of biological systems has been used in previous research on epicuticular plant wax coverage [53], animal attachment devices [54], and insect wings [55].

Taking into account our previous extensive morphological, ultrastructural, and experimental studies performed with *N. alata* [26,29–31,33,36–38,46], we selected this tropical pitcher plant species as a native prototype for our biomimetic pitchers. We defined *N. alata* pitchers as the ancestral type according to [12]. In nature, *N. alata* plants grow in mossy forests at 800–2400 m altitude on several Philippine Islands [9,11]. The plants produce both types of pitchers (lower when young and upper when mature), which do not differ much from each other.

Our working hypothesis was that a tube partly filled with fluid is able to trap insects (i.e., functions as a primitive trap), whereas the particular tube shape and inner surface coverage additionally increase the trapping efficiency. Trapping efficiency (trapping success) is defined here as number of prey items captured per tube or pitcher. To test this, we performed several sets of trapping experiments with *Drosophila melanogaster* (Meigen, 1830) flies (Diptera, Drosophilidae) and tubes (1) containing different liquids (water, Triton X-100, or Domol), (2) differing in dimensions (both height and diameter), and (3) having different microparticle coatings (calcium carbonate or kaolin). This fly species was chosen as a representative of small flying insects from the insect order Diptera, which is known to be one of the most frequently captured by the pitcher plants [4,13,17]. For the experiments, we considered that our artificial pitchers (tubes) did not function as traps if no or a very few insects were captured. Based on the results of some preliminary statistical exercises, starting from three trapped prey items equaling 1% of released insects, we assumed that these tubes can be regarded as trapping structures.

We also examined insect attachment organs in a clean condition and after flies contacted microparticle coatings. Furthermore, we estimated the wetting properties of liquids used in trapping experiments.

2. Materials and Methods

2.1. Trapping Experiments

The experiments were performed in a climate chamber (Figure 2A) with a photoperiod of 14 light hours and 10 dark hours at a constant temperature of 25 °C and a relative humidity of 60%, where plastic boxes containing test tubes were placed (Figure 2C). A plastic box (19 cm × 19 cm × 19 cm, ca. 5.9 L; BraPlast, Vittsjö, Sweden) had a top covered with a fly screen and a flap door at the front, which was used to insert insects into the box (Figure 2).

Two types of plastic (chemically inert, odorless, semitransparent polypropylene) cell culture tubes (CELLSTAR® tubes, Greiner Bio-One, Frickenhausen, Germany) were used in the experiments: (1) 30 mm in diameter and with a 50 mL volume capacity and (2) 17 mm in diameter and with a 15 mL volume capacity (Figure 3B). The tubes were cut with a rotary carver to the right height needed for the experiment (8, 9, or 10 cm) (Figure 3A), and the resulting edges were polished by hand using sandpaper. The remaining polypropylene dust was cleaned off using compressed air. Such a procedure was applied to make all testing tubes.

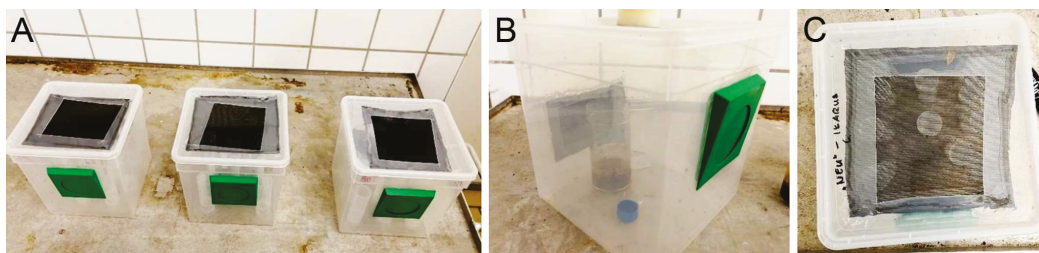


Figure 2. Plastic boxes used for experiments: boxes in the climate chamber (A); the box containing a tube used for the breeding of *Drosophila melanogaster* flies (B); the running experiment with different test tubes inside the box, view from above (C).

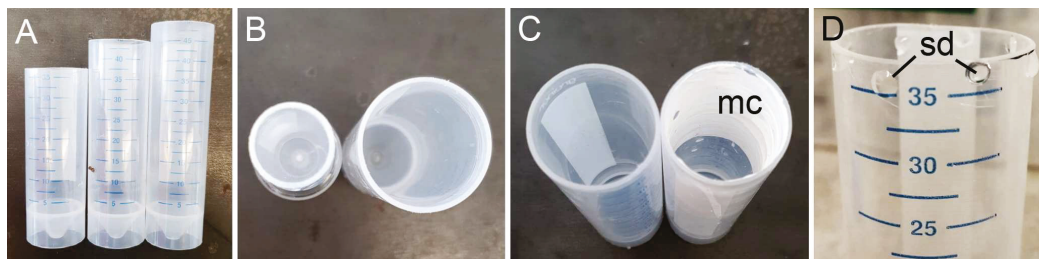


Figure 3. Plastic tubes used in the experiments: 30 mm in diameter and different heights (8, 9, and 10 cm, from left to right) (A); 10 cm high and different diameters (17 and 30 mm, left and right, respectively), view from above (B); tubes with intact (left) or modified, i.e., bearing a microparticle coating (right), inner surface, view from above (C); upper part of the tube with syrup droplets placed on its rim (D). For better visualization, the tube with a modified inner surface shown in (C) (right) has a wider strip of the coating than tubes used in the experiments. mc, microparticle coating; sd, sugar cane syrup droplets.

To attract insects to the tubes, eight small 0.1 g drops of sugar cane syrup (Riemerschmid®, Erding, Germany) mimicking the nectar were placed on each tube rim using a pipette, four on its outer side and four on the inner side (Figure 3D). In experiments with the painted tubes, in order to avoid possible damage and/or contamination of a coating by the syrup, we positioned four droplets only on the outer tube side.

Test tubes were consistently filled to 20% of their volume with a liquid imitating the pitcher digestive fluid. This was either (1) water, (2) a water mixture of Triton X-100 in a 50:1 volume ratio (later called as Triton), or (3) a water mixture of 2.4.1. Domol heavy duty detergent in a 100:1 ratio (later called as Domol). Triton (4-phenyl-polyethyleneglycol, Sigma-Aldrich, Hamburg, Germany) is a non-ionic surfactant and emulsifier. We used Triton from our old stock. However, because of its toxic effect, especially on humans and the environment, its use is now banned in the European Union (<https://echa.europa.eu/authorisation-list>, accessed on 1 April 2025). Therefore, in further experiments, we applied non-toxic Domol (Dirk Rossmann GmbH, Burgwedel, Germany), in which the main ingredients are surfactants (15–30% anionic and 5–15% non-ionic surfactants), including alkylbenzene sulfonates, sodium C12–18 sulphate, and linear alcohol ethoxylates C13 and C15.

The epicuticular wax coverage was simulated by two different microparticle coatings: (1) calcium carbonate and (2) kaolin. Three-centimeter-wide strips of the coating were applied to the upper part of the inner surface in the tube (Figure 3C). For the calcium carbonate (CaCO_3) coating, the tube was painted using a brush with slaked lime (calcium hydroxide $\text{Ca}(\text{OH})_2$, Art-Nr. KK03.1; Carl Roth GmbH, Karlsruhe, Germany) dissolved in double-distilled water (20 g lime: 25 mL water) according to [56]. After air-drying, in the presence of carbon dioxide CO_2 , a microrough layer of calcium carbonate crystals was formed on the surface. Calcium carbonate is one of the first minerals used in the so called

“ancient particle film technology” and represents insecticidal material, which has been widely applied in agriculture in the early 1900s and is still used nowadays against insect pests and plant pathogens [57]. The kaolin coating was obtained by painting the tube surface with a water suspension of kaolin powder (bolus alba = kaolin, Art-Nr. 8361.2; Carl Roth GmbH, Karlsruhe, Germany) containing mostly kaolinite, i.e., hydrated aluminum silicate $\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$, in the ratio 1:1. After drying under air conditions for 24 h, a microstructured kaolin coating was created. Kaolin particle films along with other particle films, especially those based on aluminosilicates, are intensively used in agriculture for plant protection against insect pests, particularly in the last two decades [57]. The above two coatings differed in the shape and dimensions of their particles as well as roughness; the mean roughness (R_a) was $2.16 \pm 0.44 \mu\text{m}$ for calcium carbonate vs. $1.54 \pm 0.09 \mu\text{m}$ for kaolin, and the root mean square of roughness (r.m.s.) was $2.77 \pm 0.61 \mu\text{m}$ for calcium carbonate vs. $1.93 \pm 0.01 \mu\text{m}$ for kaolin [58,59]. For comparison, the wax coverage (upper layer) in *N. alata* pitchers showed $R_a = 1.91 \pm 0.25 \mu\text{m}$ and r.m.s. = $2.38 \pm 0.30 \mu\text{m}$ [29]. All above roughness data were obtained at the same ($\times 50$) magnification using a white light interferometer.

The following experimental set-ups were realized to study different effects, such as

(a) Digestive fluid and (b) pitcher dimensions for

- Diameter:
 - (1) Tubes 17 mm vs. 30 mm in diameter (both 10 cm high) filled with water;
 - (2) Tubes 17 mm vs. 30 mm in diameter (both 10 cm high) filled with Triton;
 - (3) Tubes 17 mm and 30 mm in diameter (both 10 cm high) filled with Domol;
- Height:
 - (4) Tubes 8 cm vs. 9 cm vs. 10 cm high (all 30 mm in diameter) filled with water;
 - (5) Tubes 8 cm vs. 9 cm vs. 10 cm high (all 30 mm in diameter) filled with Triton;
 - (6) Tubes 8 cm vs. 9 cm vs. 10 cm high (all 30 mm in diameter) filled with Domol;
- (c) Coverage of the inner pitcher surface imitated by
 - Calcium carbonate:
 - (7) Tubes intact vs. painted (10 cm high, 30 mm in diameter), filled with surfactant;
 - Kaolin:
 - (8) Tubes intact vs. painted (10 cm high, 30 mm in diameter), filled with surfactant.

Nine (3 + 3 + 3) or ten (5 + 5) tubes, depending on the experimental set-up (nine in (4)–(6) and ten in (1)–(3), (7), and (8)), were put in the random order into the plastic box, where 100 flies of *D. melanogaster* (the wild type *w118* obtained from the working group of Prof. T. Roeder (Department of Molecular Physiology, Zoological Institute, Kiel University)) were released. After 24 h, the number of flies trapped by the fluid in each tube was counted. We performed three experiments (in three boxes) simultaneously for every experimental set-up (Figure 3A). For analyses, results of the three repetitions were cumulated.

The number of flies trapped in different tube types was statistically analyzed for each experimental set-up using the Chi-square test (software Good Calculator, free online calculators, <https://goodcalculators.com/>). For each test, the expected number of trapped flies in each tube type was calculated as $expected = total \text{ number of trapped flies} / \text{number of tube types}$.

To estimate whether the test tube type represented a trap, we calculated the average number of trapped flies per tube of this type for each experimental set-up: $average \text{ number of trapped flies per tube of this type} = \text{number of flies trapped by tubes of this type} / \text{number of tubes of this type}$. If this number exceeded 3 (i.e., 1% of insect individuals inserted into the 3 boxes), this tube type was considered a trapping unit.

2.2. Cryo Scanning Electron Microscopy (Cryo SEM)

In order to visualize the possible contamination of insect tarsal attachment organs by microparticles, *D. melanogaster* flies (the wingless mutant *wg/Wnt-1* obtained from Prof. T. Roeder working group) were investigated after they had been kept for 1 h in (1) a clean non-coated tube, (2) a tube bearing a calcium carbonate coating, (3) a tube with a kaolin coating, or (4) an air-dried *N. alata* pitcher used as a control. The tested flies were then shock-frozen in liquid nitrogen ($-196\text{ }^{\circ}\text{C}$) and prepared for cryo SEM examination as follows.

Insects were mounted with their backs to a holder and frozen in a cryo preparation chamber (ALTO 2500 cryo preparation system, Gatan Inc., Abingdon, UK) at $-140\text{ }^{\circ}\text{C}$. Afterwards, they were sputter coated in a frozen condition with gold-palladium (6 nm thickness) and examined at a 3 kV acceleration voltage and a temperature of $-120\text{ }^{\circ}\text{C}$ at the cryo stage within the SEM Hitachi S-4800 (Hitachi High-Technologies Corp., Tokyo, Japan).

Additionally, the surfaces of both coatings and air-dried waxy zone of *N. alata* pitchers obtained from live plants growing in the greenhouse of the Botanical Garden at Kiel University were studied using the same cryo SEM approach.

2.3. Contact Angle (CA) Measurements

CAs of four different liquids (double-distilled water, Domol-water mixture, and Triton-water mixture used in the experiments as well as the digestive fluid from *N. alata* pitcher as a reference) were measured on five surface samples (smooth hydrophilic glass and hydrophobic Teflon, microstructured calcium carbonate and kaolin coatings, and the waxy zone of *N. alata* pitcher bearing the epicuticular wax coverage as a control) using a high-speed optical CA measuring device OCAH 200 (DataPhysics Instruments GmbH, Filderstadt, Germany). We were primarily interested in the wetting properties of liquids used in the trapping experiments. The digestive fluid and pitcher samples were obtained on the day of measurements from live plants growing in the greenhouse of the Botanical Garden at Kiel University. We used $0.5\text{ }\mu\text{L}$ sessile drops and circle-fitting for the evaluation of static CAs. With each liquid on each surface, 10 measurements were performed. Data are presented in the text as the mean $\pm$ standard deviation.

Data on CAs obtained on different surfaces for each liquid were statistically analyzed using one-way ANOVA (for water and Triton) or Kruskal–Wallis one-way ANOVA on ranks (for Domol and the pitcher fluid) (SigmaStat 3.5, Systat Software Inc., Point Richmond, CA, USA), followed by the post hoc Holm–Sidak method (for water and Triton), Tukey test (for Domol) and Dunn’s method (for the pitcher fluid) for pairwise comparisons between samples. Data are presented in the corresponding figures as box-and-whisker graphs (https://en.wikipedia.org/wiki/Box_plot, accessed on 13 February 2026), where the box is drawn from the lower quartile Q_1 (the median of the lower half of the dataset) to the upper quartile Q_3 (the median of the upper half of the dataset) with a horizontal line inside it denoting the median Q_2 (the middle value in the dataset). The whiskers are based on the 1.5 IQR value ($\text{IQR} = Q_3 - Q_1$): from Q_3 , a distance of 1.5 times the IQR is measured out and a whisker is drawn up to the largest observed data point from the dataset that falls within this distance; similarly, a distance of 1.5 times the IQR is measured out below the Q_1 and a whisker is drawn down to the lowest observed data point from the dataset that falls within this distance. All other observed data points outside the boundary of the whiskers are plotted as outliers.

3. Results

3.1. Trapping Efficiency of Differently Prepared Tubes

Results of the trapping experiments performed according to eight different set-ups (1–8) (see Section 2. Materials and Methods, Section 2.1. Trapping experiments) are presented in Table 1.

Table 1. Results of trapping experiments performed with different tube types using eight set-ups.

Set-Up	Tube Type	Liquid	Tested Character	Categories	Trapped Total ¹	Trapped Per Tube ²	Trapped Per Tube (%) ³
1	10 cm high	water	diameter	17 mm 30 mm	3 23	0.20 1.53	0.07 0.51
2	10 cm high	Triton	diameter	17 mm 30 mm	59 240	3.93 16.00	1.31 5.36
3	10 cm high	Domol	diameter	17 mm 30 mm	40 241	2.67 16.07	0.89 5.36
4	30 mm diameter	water	height	8 cm 9 cm 10 cm	3 5 1	0.33 0.56 1.11	0.11 0.19 0.04
5	30 mm diameter	Triton	height	8 cm 9 cm 10 cm	52 61 129	5.78 6.78 14.33	1.93 2.26 4.78
6	30 mm diameter	Domol	height	8 cm 9 cm 10 cm	97 86 108	10.78 9.56 12.00	3.59 3.19 4.00
7	10 cm high, 30 mm diameter	Triton	calcium carbonate coating	no yes	125 175	8.33 11.67	2.78 3.89
8	10 cm high, 30 mm diameter	Domol	kaolin coating	no yes	63 233	10.50 38.83	3.50 12.94

¹ Trapped total is the number of *Drosophila melanogaster* individuals trapped by all tubes of this type in three experimental boxes. ² Trapped per tube is an average number of trapped flies per tube of this type (calculated as number of flies trapped by all tubes of this type divided by number of tubes of this type). ³ Trapped per tube (%) is an average number of trapped flies per tube of this type calculated in relation to 300 fly individuals initially inserted into the 3 boxes.

3.1.1. Effects of the Fluid and Tube Dimensions

In tubes filled with water (set-ups (1) and (4)), a very low number (less than 3 individuals per tube) of *D. melanogaster* flies were trapped. The highest amount of prey reaching in average 1.5 per tube was recorded in 10 cm high and 30 mm wide tubes (set-up (1)). In all, only 9 and 26 trapped flies (set-ups (4) and (1), respectively) were registered in these two experiments. However, at least one insect individual was trapped by each tested tube type.

In the case of water, tube dimensions have different effects on the trapping efficiency. Tubes with different heights (8, 9, and 10 cm, set-up (4)) captured a similar amount of prey ($\chi^2 = 2.6660$, d.f. = 2, $p = 0.2636$), whereas there was a statistically significant difference ($\chi^2 = 15.3846$, d.f. = 1, $p < 0.0001$) between tubes having different diameters (set-up (1)): 30 mm wide tubes trapped more flies than 17 mm tubes.

Compared to water-filled tubes, unpainted tubes filled with Triton demonstrated a higher quantity of trapped flies: 242 and 299 (set-ups (5) and (2), respectively) for every time 300 released flies were trapped in these two experiments. For each tube type, much more than three insects per individual tube, on average, were captured. The highest number of flies (14 and 16 in average in the set-ups (5) and (2), respectively) were trapped in 10 cm high and 30 cm wide tubes.

Both the length and diameter in clean, unpainted, Triton-filled tubes showed highly significant differences in terms of trapped insects: $\chi^2 = 43.7654$, d.f. = 2, $p < 0.0001$ for height (set-up (5)); $\chi^2 = 109.2067$, d.f. = 1, $p < 0.0001$ for diameter (set-up (2)). Among three different heights, the significantly best trapping success was achieved by the highest (10 cm high) tubes ($p < 0.0001$ for both comparisons), whereas shorter tubes (8 and 9 cm)

did not differ from each other ($p = 0.3964$). In the case of diameter, better results were found in wider tubes.

Unpainted tubes filled with Domol trapped more *D. melanogaster* flies compared to those containing Triton and especially in comparison with water-filled ones; more than 280 flies were captured in each experiment. These tubes trapped on average from 9.5 (9 cm high and 30 cm in diameter, set-up (6)) to 16.0 insects (10 cm high and 30 mm in diameter, set-up (3)) per tube. The only exception was 17 mm wide tubes (set-up (3)), in which less than three insects (2.6 in average) were trapped.

The statistical comparisons between effects of the height and diameter in unpainted, Domol-filled tubes showed similar results to those found in water-filled tubes. Tubes of different heights (set-up (6)) trapped a similar amount of flies ($\chi^2 = 2.4948$, d.f. = 2, $p = 0.2872$), while tubes having different diameters (set-up (3)) demonstrated significantly different trapping efficiencies ($\chi^2 = 143.2695$, d.f. = 1, $p < 0.0001$), where 30 cm wide tubes were much more efficient.

3.1.2. Effect of Microparticle Coatings

In both experiments with surfactant-containing tubes having microparticle coatings inside, all (calcium carbonate, set-up (7)) or nearly all (kaolin, set-up (8)) of 300 flies were captured. For both coatings, quantities of trapped insects essentially exceeded an average of three individuals per tube in both tube types tested (painted and unpainted), with lower numbers in unpainted tubes and higher numbers in painted ones.

Statistical analyses of fly numbers captured in different tube types detected significant differences between unpainted and painted tubes in both experiments with coatings: $\chi^2 = 8.3334$, d.f. = 1, $p < 0.05$ for calcium carbonate (set-up (7)) and $\chi^2 = 97.6352$, d.f. = 1, $p < 0.0001$ for kaolin (set-up (8)). Interestingly, when we compared the portions of trapped insects in unpainted and painted tubes, these values differed between coatings: it was 1.0:1.4 in experiments with calcium carbonate and 1.0:3.7 in experiments with kaolin.

3.2. Contamination of Insect Tarsal Attachment Organs by Different Microparticle Coverages

Attachment organs (both claws and two hairy adhesive pads (pulvilli) bearing tenent setae) of *D. melanogaster* flies kept in non-coated tubes remained clean after the experiment (Figure 4A,B).

The calcium carbonate coating represented a rather loose coverage composed of separate flower-shaped groups, like rosettes, of cone-shaped structures, which were oriented at various angles to the underlying surface (Figure 5A,B). Contact with this coating inside the tubes led *D. melanogaster* to very little contamination of the adhesive pads, in a few cases (Figure 4C,D). If present, the contaminating material was located mainly on shafts of tenent setae or stuck between them. Weak contamination was also visible on claws (Figure 4C). These contaminating particles had irregular compact shapes.

The kaolin coating was also not compact and consisted of several superimposed layers of polygonal platelets placed parallel to the underlying surface (Figure 5C,D). This coating caused rather strong contamination of both claws and adhesive pads by relatively big flattened patches (Figure 4E). In addition to setal shafts and bases, spatula-shaped setal tips were dirty (Figure 4F). In some cases, setal tips stuck together due to the contaminant between them.

The surface of the epicuticular wax coverage of the slippery zone inside the *N. alata* pitcher had a rather uniform appearance due to densely and regularly placed, perpendicularly protruding platelet-shaped wax projections (Figure 5E,F). The experiments with *N. alata* wax coverage resulted in a very heavy contamination of the whole fly foot with flat

wax particles (Figure 4G,H), where the adhered wax material often caused the conglutination of the setae (Figure 4H).

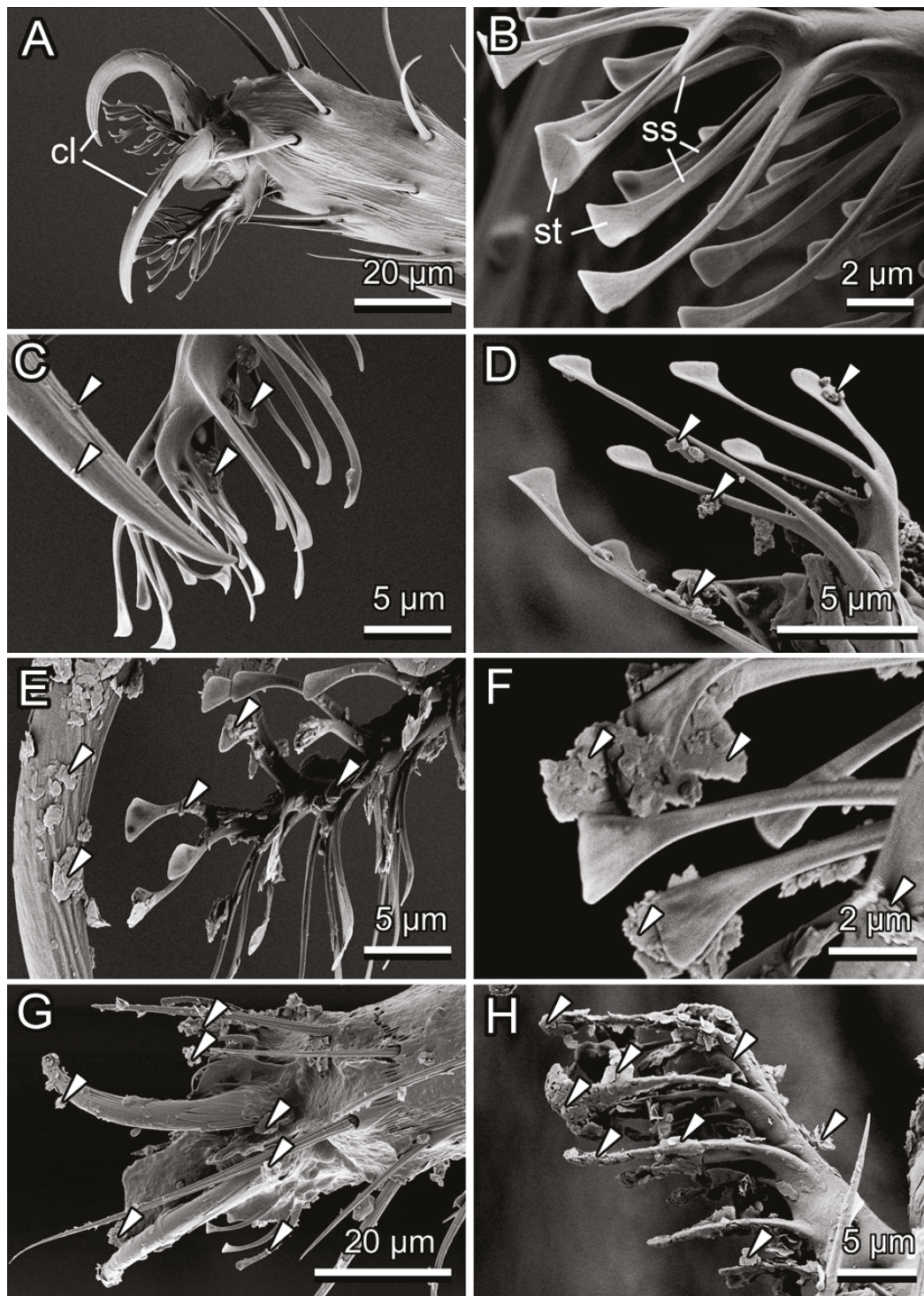


Figure 4. Cryo SEM images of *Drosophila melanogaster* attachment organs after experiments on clean tubes (A,B), tubes bearing the calcium carbonate (C,D) and kaolin (E,F) coatings, and air-dried pitchers of *Nepenthes alata* (G,H). White arrowheads point to the contaminating material. cl, claw; ss, shaft of tenent seta; st, tip of tenent seta.

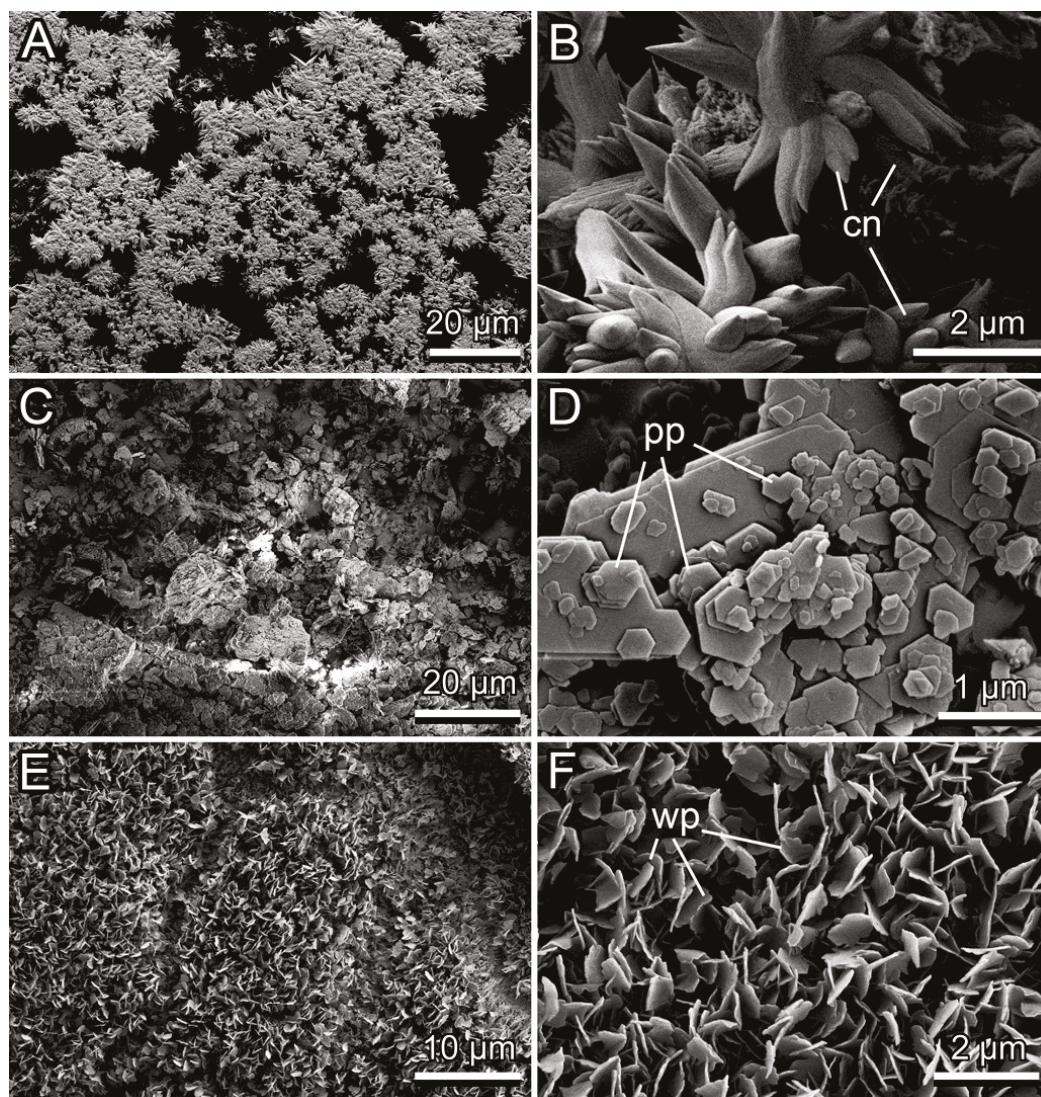


Figure 5. Cryo SEM images of the surfaces with the calcium carbonate (A,B) and kaolin (C,D) coatings and the epicuticular wax coverage inside the *Nepenthes alata* pitcher (E,F). cn, cone-shaped structure; pp, polygonal platelet; wp, wax platelet.

3.3. CAs of Tested Liquids on Different Surface Samples

In cases of both microparticle coatings (calcium carbonate and kaolin), all tested liquids were immediately sucked in, i.e., absorbed. In these cases, we regarded CAs as 0°.

Three liquid-surface-air systems showed CAs exceeding 90° (Figure 6). These were water on the waxy pitcher surface of *N. alata* ($103.84 \pm 5.96^\circ$), water on Teflon ($100.83 \pm 5.70^\circ$), and the digestive fluid of the pitcher on the waxy pitcher surface of *N. alata* ($95.09 \pm 11.65^\circ$). In other tested systems, CAs ranged on average from around 7° (Triton on glass) to almost 59° (the digestive fluid on Teflon).

Figure 7 shows the wetting properties of four tested liquids on three solid surface samples. Water demonstrated significantly different CAs on the test surfaces (one-way ANOVA, $F = 461.815$, d.f. = 2, $p < 0.001$). It readily wetted glass samples, whereas Teflon and the waxy pitcher surface had significantly similar hydrophobic properties (Holm-Sidak method: glass vs. Teflon and glass vs. pitcher, both $p < 0.05$; Teflon vs. pitcher, $p = 0.253$). Triton and Domol showed similar wetting properties; all tested surfaces were wetted by both liquids, however, with significantly different CAs (one-way ANOVA for Triton, $F = 131.346$; Kruskal–Wallis one-way ANOVA on ranks for Domol, $H = 25.806$; both d.f. = 2, $p < 0.001$): the lowest CAs on glass, the highest ones on Teflon, and the intermediate

ones on the pitcher surface (Holm-Sidak method for Triton and Tukey test for Domol, $p < 0.05$ in all comparisons). As for the digestive fluid, both glass and Teflon were wetted, although with significantly different CAs, whereas the waxy pitcher zone was unwettable (Kruskal–Wallis one-way ANOVA on ranks: $H = 26.673$, d.f. = 2, $p < 0.001$; Dunn’s method, $p < 0.05$ in all comparisons).

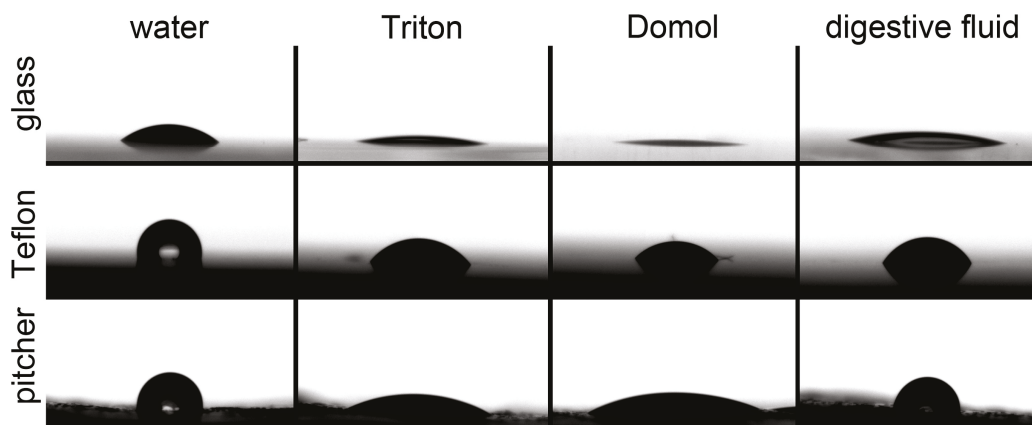


Figure 6. Exemplary 0.5 μL droplets of four liquids (water, Triton, Domol, and the digestive fluid of the *Nepenthes alata* pitcher) on three surface samples (glass, Teflon, and the waxy surface of the *N. alata* pitcher).

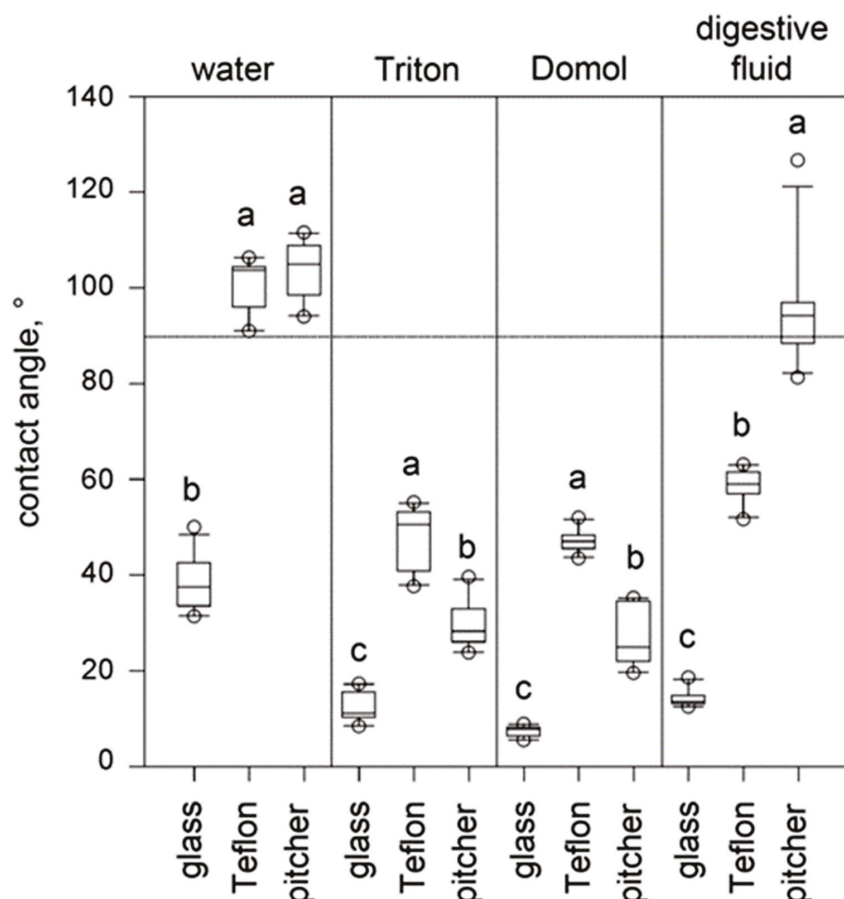


Figure 7. Wetting properties of four different liquids. Boxplots show the interquartile range and the medians, whiskers indicate the $1.5\times$ interquartile range, and “o” are outliers. Data represented by boxplots with different letters within one liquid are significantly different at $p < 0.05$. A dashed line marks 90° .

Figure 8 shows the wettability of three solid surface samples by four different liquids. Glass was well wetted by all used liquids, much better than Teflon and the pitcher surface (Kruskal–Wallis one-way ANOVA on ranks for all, $H = 117.421$, d.f. = 11, $p < 0.001$; Dunn's method for glass vs. Teflon and glass vs. pitcher surface, $p < 0.05$ in all comparisons). The latter two surfaces demonstrated the same hydrophobic properties (Dunn's method, $p > 0.05$) and were rather similar, although with significantly different CAs, in cases of Triton and Domol (Dunn's method, both $p < 0.05$) but showed completely different behaviors in regard to the pitcher fluid; Teflon was wettable ($58.90 \pm 3.32^\circ$), whereas the pitcher surface was unwettable.

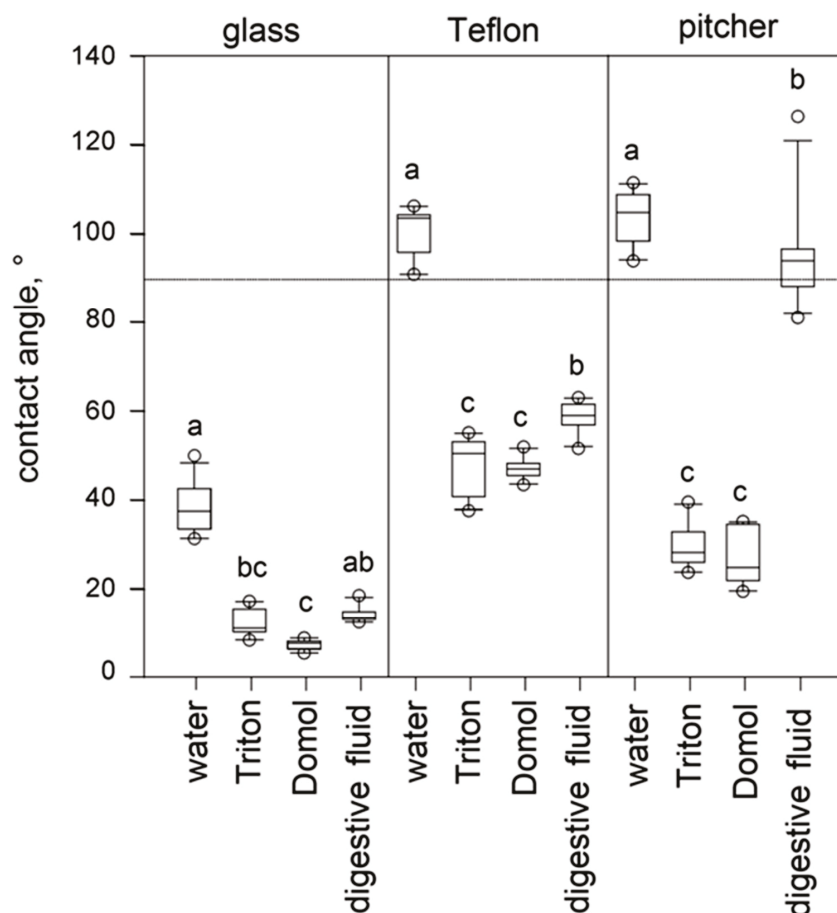


Figure 8. Wettability of three surface samples. Boxplots show the interquartile range and the medians, whiskers indicate the $1.5 \times$ interquartile range, and “o” are outliers. Data represented by boxplots with different letters within one surface sample are significantly different at $p < 0.05$. A dashed line marks 90° .

4. Discussion

4.1. Effects of the Tube Dimensions, Inner Surface Coatings and Liquids on Insect Trapping Efficiency

Our trapping experiments clearly demonstrated that the type of liquid has a more important influence on the trapping efficiency compared to different tube dimensions. Although all tested tubes of different sizes containing water as an analogy to the pitcher fluid captured a very small number of insects, Domol-filled and especially Triton-filled tubes served as trapping structures capturing (much) more than 1% of released flies. Our CA measurements showed that in contrast to water, both surfactant-containing liquids (Triton and Domol) showed similar, strong wetting properties; they readily wetted all tested surfaces, among them hydrophobic Teflon and the waxy pitcher surface. Interestingly,

the digestive fluid from *N. alata* pitchers demonstrated similar properties in the case of hydrophilic glass, still wetted Teflon, though at a higher contact angle, but was not able to wet the pitcher's waxy surface. From results of our CA measurements, we could conclude that both Triton and Domol were liquids with similar physicochemical properties to the plant liquid and therefore proper to use as biomimetic fluids in our trapping experiments.

Our results on CA of water on the *N. alata* waxy pitcher zone are in line with those obtained in the previous investigation on the wettability of different inner pitcher surfaces in this plant species [37], where the waxy zone showed superhydrophobic properties and was also very poor wetted (CAs $> 120^\circ$) by two other liquids, such as extremely non-polar diiodomethane (surface tension = 50.0 mN/m, dispersion component = 47.4 mN/m, polar component = 2.6 mN/m [60]) and polar ethylene glycol (surface tension = 48.0 mN/m, dispersion component = 29.0 mN/m, polar component = 19.0 mN/m [61]). It has also been previously demonstrated in the experiment with spraying water over the waxy zone of *N. alata* pitchers that water forms separate droplets having a very high contact angle with the surface [26,38].

The digestive fluid in *Nepenthes* pitchers contains many digestive enzymes, antimicrobial compounds, mineral nutrients, and acidic polysaccharides [47,52,62]. The role of this fluid in carnivorous pitcher plants has been mainly studied in terms of its contribution to prey utilization, namely its chemical function in digestion, absorption, and transport of the prey-derived nitrogen compounds. As for its trapping function, it has been suggested that the pitcher fluid in *N. alata* does not act as a paralyzing substance like it has been previously demonstrated for *Sarracenia flava* L. (Sarraceniaceae) pitchers [4] but rather as a kind of glue that impedes insects' locomotion [30]. Later, it was found first in *N. rafflesiana* and then also in *N. ampullaria* Jack, *N. gracilis*, and *N. hemsleyana* Macfarl. that this fluid exhibits a good wetting capability and a low surface tension; it wets an insect as soon as motion occurs and makes escaping from the pitcher almost impossible [47,49]. In several studies, the trapping success of the digestive fluid has been concatenated mainly to its viscoelastic properties, especially its high viscosity [12,15,47,63]. It has been reported that pitchers of many other *Nepenthes* species produce digestive fluid having similar physicochemical properties [48,64]. Especially in wax-lacking *Nepenthes* species, the digestive fluid was found to be particularly viscous [64,65]. According to [17,49], also the acidic pH of the fluid contributes to prey capture. The recent experimental study revealed that (1) insects sink more easily in the *N. rafflesiana* pitcher fluid than in water due to its reduced surface tension, (2) within the pitcher fluid, more energy is required to separate insects from the fluid than from water, and (3) the fluid prevents dewetting and makes it difficult for insects to free themselves [66]. Thus, based on the results of various studies, the digestive fluid was considered to induce the trapping and retention of prey in *Nepenthes* pitchers. In our trapping experiments, two used liquids (Domol and Triton) contained surfactants and, therefore, were able to readily wet *D. melanogaster* flies. At least owing to these wetting properties, both test liquids essentially contributed to insect capture, in contrast to water.

The wetting behavior of tested liquids closely reflects their surfactant composition. Water, lacking surfactants, exhibited high contact angles on hydrophobic surfaces such as Teflon and the waxy pitcher zone, whereas Triton and Domol, containing non-ionic and anionic surfactants, readily spread across all surfaces, reducing contact angles and mimicking the low surface tension characteristic of natural digestive fluids. Notably, the digestive fluid of *N. alata*, despite being aqueous, displayed selective wettability (wetting hydrophilic surfaces while the waxy pitcher zone remained unwet), demonstrating that its complex composition of surface-active compounds achieves a combination of adhesion, slipperiness, and prey capture not fully replicated by simple surfactants.

The experimental data showed a certain difference between the effects of height vs. diameter of the tubes on their trapping function. Filled with water, tubes of different heights were equally inefficient in trapping, whereas wider tubes captured significantly more flies than narrower ones. In the case of Triton, both the height and diameter contributed to the prey capture: higher and wider tubes demonstrated significantly stronger trapping success. With Domol-filled tubes, we obtained similar results to those found in water-filled tubes, however, with a much higher number of trapped insects. These effects, which were especially pronounced in experiments with Triton-filled tubes, may have the following explanations. In the case of higher/slender tubes, the probability that insects fly out and escape is much lower because of the relatively high walls of the trap. The fact that we did not see the effect of the tube height in our tests with water- and Domol-filled tubes may be a result of a rather small range (8–10 cm) of the used variability. Possibly, when we apply stronger differences in height (i.e., 5, 7.5 and 10 cm), we would obtain more distinct differences in prey capture between tube types. As to the impact of the diameter, it may be explained by a straightforward geometrical effect: more insects could get into a wider trap. Previously, in a study with *Nepenthes* plants, it has been shown that pitchers having a larger diameter of pitcher opening captured more flying insects than those with a narrower one [17]. Our results obtained with wide tubes are to a certain extent in line with this finding. The previous authors suggested that fluid-filled funnel-shaped pitchers with a large diameter probably act as reflection–polarization traps attracting flying insects [17]. Since our experiments were performed with cylindrical tubes and the distance between the fluid level and tube opening was relatively long, we doubt whether a large diameter can make the fluid more visible to insects in this case.

Although most *Nepenthes* species are known to vary in their prey composition, as they are usually not specialized in one species of prey but rather catch different arthropods in general [20], their pitchers still differ from each other in size, shape, and color pattern [52], depending not only on the plant species (Figure 9) but also on the location on the plant. Generally, when pitcher dimorphism is present, the lower pitchers are smaller, more compact, and overall wider than the upper pitchers, which prevalently prey flying insects, as described, for example, in *N. rafflesiana* [13] and *N. madagascariensis* Poir. [67]). In these cases, the lower pitchers better accommodated non-flying insects and provided more space for them to crawl into. Recently, in a study on seven *Nepenthes* species, it has been demonstrated that pitcher volume and diameter did not differ significantly between upper and lower pitchers, whereas upper pitchers, where trapped flying insect were more abundant, were, on average, higher and had a more conical shape [17]. Interestingly, the number of ants trapped in pitchers was similar in both pitcher types.

Nepenthes alata, which served as a kind of native prototype for our “biomimetic” pitchers, is a highly polymorphic climbing plant species, with pitchers varying in color (from green to red) and dimensions reaching 25 cm in height and 6 cm in diameter. Although shape does not change much between the lower and upper pitches, there are some differences in size; close to the ground, the pitchers are usually smaller (up to 18 cm high), whereas the upper ones reach 25 cm in height. In our trapping experiments, we only slightly varied the height of the tubes (8, 9, and 10 cm) and, therefore, probably did not detect any effect of the tube height in most experimental set-ups. As for the pitcher diameter, it does not vary in *N. alata*, but this can be observed in other *Nepenthes* species [65]. For this reason, in order to understand the influence of the diameter on prey capture, we used narrow (17 mm in diameter) and wide (30 mm in diameter) tubes in our study. Our experimental results clearly demonstrated that wider tubes are more efficient in trapping.



Figure 9. Longitudinally dissected pitchers of different *Nepenthes* taxa, view from inside: *N. alata* (A), *N. fusca* Danser (B), *N. macrophylla* (Marabini) Jebb and Cheek (C), *N. mirabilis* (Lour.) Druce (D), *N. rafflesiana* (E), *N. dicksoniana* Lindsay (horticultural hybrid of *N. rafflesiana* and *N. veitchii* Hook. f.) (F), *N. ventricosa* Blanco (G), *N. veitchii* (H), and *N. bicalcarata* Hook. f. (I). A dashed line denotes the length of the slippery (waxy) zone. Scale bars: 1 cm. Adapted from [34].

In contrast to previous conclusions that cylindrical pitchers with waxy walls are particularly efficient for ants' capture in comparison to termites' and flying insects' capture [17] and wax only apparently is efficient for trapping ants [48], our experiments clearly demonstrated the effect of microscopic surface coverage on trapping flying insects. The use of a microparticle coating that simulated the epicuticular wax coverage inside the pitcher revealed its significance for the trapping function in the cases of both types of coatings (calcium carbonate and kaolin) and both surfactant-containing liquids (Triton and Domol). In these experiments, all or almost all inserted *D. melanogaster* flies were trapped. Interestingly, both intact and painted (i.e., bearing a coating) tubes successfully captured insects, however, in different proportions depending on the experimental set-up, namely the type of the coating and the type of the liquid; the kaolin coating in Domol-filled tubes trapped relatively more flies in comparison to calcium carbonate coating in Triton-filled tubes. The above microparticle coatings created a certain "critical" microroughness of the surface, which decreased the contact area between the surface and insect adhesive pads dramatically, leading to a reduction of attachment forces (according to [68–70]) and resulting in a higher trapping efficiency of coating-bearing tubes. Such an effect of the contact area reduction has been previously shown for the epicuticular wax coverage in native pitchers of some *Nepenthes* species [30,33,35]. Moreover, contamination of fly adhesive pads by microparticles detached from the coatings could additionally decrease the attachment force and contribute to the trapping efficiency of the test tubes (see below).

4.2. Effect of Contamination of Insect Attachment Organs by Microparticles

Obtained differences in the contaminating effects of different surface coatings (calcium carbonate, kaolin, and *N. alata* epicuticular wax coverage) were caused primarily by differences in dimensions and shapes of particles composing these coatings. The calcium

carbonate coating consists of relatively big, coarse, and robust surface structures, which did not readily detach from the surface under the weight of tiny *D. melanogaster* flies. These particles being rather voluminous and compact did not adhere well to insect adhesive setae and, therefore, did not produce strong contamination of insect adhesive organs. As kaolin particles are smaller and have a flattened shape (resembling wax projections found in a number of *Nepenthes* species [30,33,34]), they detached easily from the substrate, adhered to and heavily contaminated fly setal tips, which are known to be responsible for contact formation during the attachment of insects to smooth or microrough substrates [68]. Similar results for the contaminability of both calcium carbonate and kaolin coatings were obtained previously in the experimental study with the southern green stinkbug *Nezara viridula* (Linnaeus, 1758) (Hemiptera: Pentatomidae) and the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae), in which these coatings caused a strong reduction of the insect attachment ability mainly due to the contamination of adhesive pads [58,59]. Interestingly, both contaminating and insect attachment reduction effects were much more pronounced in the case of kaolin. The pear psylla *Cacopsylla pyricola* (Foerster, 1848) (Hemiptera: Psyllidae) also demonstrated a significantly decreased ability to hold onto plant surfaces covered with different types of kaolin coatings [71].

A heavy contamination of *D. melanogaster* attachment organs and especially adhesive pads was observed in our tests with the wax coverage of *N. alata* pitchers bearing microscopic platelet-shaped wax particles. The contaminating ability of *Nepenthes* wax has been repeatedly reported previously [4,21,30,33,36] including the study with *D. melanogaster* flies [36]. The reduction of insect attachment caused by contamination of their adhesive pads with epicuticular plant wax projections has been revealed by both experimental [72,73] and theoretical studies [74,75], showing that this effect is especially pronounced in plant surfaces covered with wax projections having higher aspect ratios.

5. Conclusions

Our trapping experiments with differently prepared tubes serving as biomimetic pitchers clearly demonstrated that the type of liquid had the most important impact on the trapping function: surfactant-containing liquids having strong wetting properties in contrast to water, ensured high success in trapping *D. melanogaster* flies. The diameter of the trap rather than its height, which had a relatively small range in our study, affected the trapping efficiency, with wider tubes capturing more insects than narrower ones, probably by providing a larger space for more insects to get into traps. The presence of a microparticle coating mimicking the epicuticular wax coverage inside the pitcher, which is characteristic of numerous *Nepenthes* species, additionally contributed to the high capture achievement, not only due to reduced contact between the trap surface and insect feet but also because of the contamination of insect attachment organs by the particles detached from the coating.

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Abbreviations

The following abbreviations are used in this manuscript:

CA Contact angle
SEM Scanning electron microscopy

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Article

Hydraulic Asymmetries for Biological and Bioinspired Valves in Tubular Channels: A Numerical Analysis

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Abstract

Biological, biomimetic, and engineering systems make extensive use of hydraulic asymmetries to control flow inside tubular structures. Examples span physiological valves, the guided transport observed in shark intestines, and passive devices such as Tesla valves. Here we investigate the mechanisms that generate these asymmetries using the notion of diodicity, defined as the ratio between pressure drops required to drive the same flow in opposite directions. We first focus on 2D geometries, which allow us to identify and study the main contributions to hydraulic asymmetry: channel geometry and internal obstacles embedded within a channel with rigid walls. By considering both rigid and deformable obstacles, we model channels that always remain open in both directions and channels that can be completely blocked by valve-like structures. We then extend the analysis to 3D geometries, again considering rigid and elastic cases. As a general trend, we find that geometry alone establishes a baseline diodicity, while higher dimensionality and structural reconfiguration consistently amplify the effect.

Keywords: Tesla valve; bioinspired; diodicity; computational fluid dynamics; COMSOL Multiphysics; numerical simulation

1. Introduction

In many natural and engineered systems, fluid flow through a channel depends on the direction of motion. The same geometry can offer low resistance in one direction and much higher resistance in the opposite one. We refer to this directional contrast in hydraulic response as hydraulic asymmetry.

Biological organisms make extensive use of this principle. A clear example is provided by heart valves made of thin leaflets that swing open under the forward pressure pulse and fold closed as soon as the pressure drops, sealing the passage and preventing significant backflow. Similarly, the lymphatic system contains intraluminal leaflet valves that function to bias lymph flow back towards the heart [1]. Exploiting the same concept with a different strategy, sharks achieve hydraulic asymmetry without occlusion in their digestive system. The intestines of sharks contain a segment called the spiral intestine, which consists of a tubular structure with internal spiral and funnel-like segments. Interestingly, this structure was demonstrated to steer the flow preferentially along the digestive path and hinder motion in the opposite direction (Figure 1). This mechanism is reproduced numerically

in Figure 2, where streamlines in a channel with a rigid spiral obstacle inspired by the intestine differ markedly between forward and reverse flow. Experimental exploration of simplified spiral-intestine-like structures demonstrates that this geometry indeed induces hydraulic asymmetry, and highlights the effect of channel deformability [2].

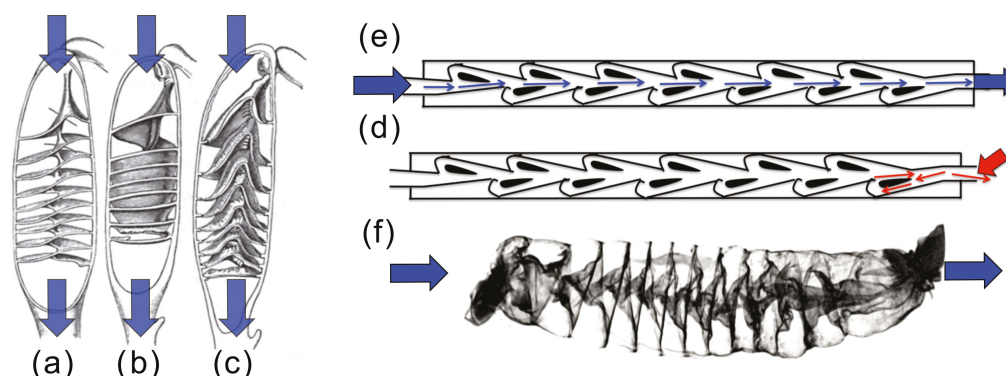


Figure 1. On the left, sketches of shark intestine representing different geometries: (a) columnar, (b) funnels pointed posteriorly, and (c) funnels pointed anteriorly. The three geometries represent different designs generating hydraulic asymmetry. Blue arrows indicate the preferential direction. On the right, a direct comparison with a Tesla valve: flux in forward direction occurs preferentially along the central axis (blue arrow) (e), while flux in the opposite direction (red arrow) involves significant recirculation (d). The similarity between this device and the shark intestine is clearly visible in (f). Images taken from [3].

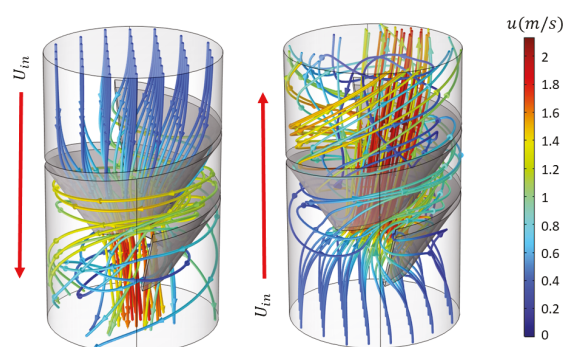


Figure 2. Numerical streamlines of flow through a channel containing a spiral obstacle inspired by shark intestines. Differences between forward and reverse flow directions demonstrate the inherent hydraulic asymmetry.

Engineered devices reproduce these ideas, often inspired by nature. Tesla valves [4] guide the flow through branches and bends that, as in the shark intestine, favor one direction over the other without any moving parts. Recent Tesla-valve applications are reviewed in [5]. Tesla-type channels have been used to suppress backflow in micro- and nanofluidic pumping architectures [6–8], and to enhance mixing by exploiting their reverse-flow response [9,10], including a multistage food-science demonstration (litmus reagent and vinegar) [11]. Related layouts have been proposed for thermal management, such as lithium-ion battery cooling [12], and for energy devices such as fuel cells [13] and photo-bioreactors [14]. These studies include numerical simulations to calculate and optimize diodicity values (see [15–18]).

Deformable channels have been considered in the literature, especially with reference to the possibility of tuning the diodicity properties by applying variable external stresses to the conduits [19]. For further work on deformable channels for microfluidic applications, we also refer the reader to [20,21].

Beyond static channels, hydraulic asymmetries can generate propulsion. In the Feynman sprinkler (Figure 3), expulsion and suction involve different streamline patterns [22]. A similar mechanism operates in the Putt-Putt boat: asymmetric expulsion and re-entry of water through the same tubes lead to unequal forces on the fluid and, hence, net thrust [23].

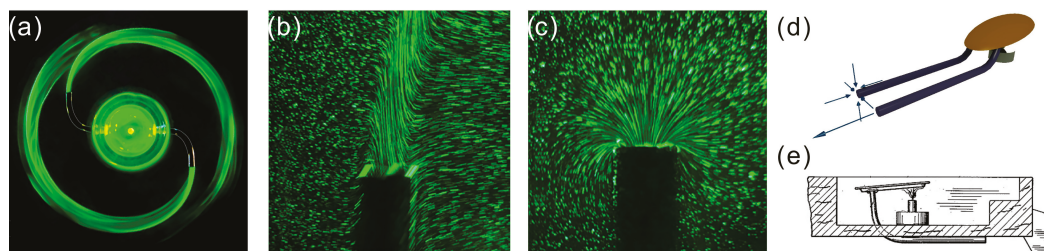


Figure 3. (a–c): Feynman sprinkler [22]. (a): Streakline photograph of the sprinkler operating in forward mode, using fluorescein dye. (b): Detail of the emitted jet and the resulting path lines near the outlet in forward operation. (c): Detail of the suction flow near the outlet in reverse operation. (d,e): Putt-Putt boat. (d): Back view of the chamber and exhaust tubes illustrating the expulsion–suction cycle. (e): Schematic diagram of a diaphragm-type Putt-Putt engine (based on a 1934 patent by Paul Jones), where asymmetric expulsion and re-entry of water generate net thrust. In both systems, the streamline topology differs between the two directions, illustrating a geometric origin of hydraulic asymmetry. Panels (a–c) reproduced and adapted from [22], with permission of the authors.

Across all these biological and technological examples, the same feature emerges: the same pressure difference can generate different flow rates and streamline patterns when the flow is reversed. We refer to this lack of odd symmetry as hydraulic asymmetry. By contrast, odd symmetry is to be expected for flows in undeformable domains at very low Reynolds numbers, because of the linearity of the governing Stokes equations, and for flows in undeformable domains with fore-aft symmetry.

The goal of this paper is to contribute to the quantitative understanding of the mechanisms underlying hydraulic asymmetry. In particular, we aim to clarify how much asymmetry can arise from geometry alone, how deformable obstacles modify or amplify it, and whether trends observed in simplified 2D settings persist in three-dimensional channels.

The key contributions and main findings of this work are as follows. First, in rigid channels with obstacles that are not fore-aft symmetric, inertial effects lead to different geometries of the streamlines when the flow direction is reverted. Thus, geometry alone is sufficient to establish baseline levels of hydraulic asymmetry. Second, larger differences in forward versus backward flow are generated by deformable obstacles, as elastic compliance introduces deformations that are sensitive to the direction of the flow. Third, in the case of deformable obstacles, the effective minimum opening under load is the key control parameter, reflecting the nonlinear balance between hydrodynamic forcing and elastic resistance. Finally, the basic 2D mechanisms for hydraulic asymmetries persist in 3D, although we were unable to reproduce in shark-intestine-inspired geometries the dissipative interactions between primary and secondary flows that are present in Tesla valves and contribute to their hydraulic asymmetry.

Previous quantitative studies of hydraulic asymmetry have largely focused on specific device classes, such as engineered fluidic diodes or individual biological systems. While these approaches have provided valuable insights, their device-specific modeling choices and performance metrics hinder direct comparisons of the roles played by geometry, deformability, and three-dimensionality across systems. Overall, we show that hydraulic asymmetry is primarily controlled by geometry and effective aperture, with material properties influencing the response through the geometries they select under load.

2. Materials and Methods

The central quantity used to characterize flow asymmetry is the diodicity D , defined as the ratio between the average pressure drops required to generate the same mean flow rate $\bar{Q}$ in the forward (ΔP^+) and reverse (ΔP^-) directions:

$$D \equiv \frac{\Delta P^+}{\Delta P^-} \quad \text{for a prescribed flow rate } Q = \bar{Q}. \quad (1)$$

This definition is appropriate when the channel never occludes, as in shark intestines, where a steady flow can be established in both directions. However, biological valves may operate in a regime of complete blockage, where the requirement of sustaining a prescribed flow rate cannot always be satisfied. In such cases, the flow rate in one direction may approach zero and the definition in (1) becomes impractical. It is then more informative to consider the flow rate generated in response to a prescribed pressure drop:

$$Q^\pm = Q(\Delta P^\pm), \quad (2)$$

This describes how much flux in the forward (Q^+) and backward (Q^-) direction the channel allows. Full blockage occurs when $Q = 0$ in one direction.

2.1. Model Overview

The analysis relies on three numerical models. The baseline configuration is a two-dimensional channel with a single obstacle placed at mid-length, analyzed first as rigid and then as free to deform under hydraulic load. A second two-dimensional model, represents a valve-like configuration in which complete blockage may occur and the alternative description (2) becomes necessary. Both models share the same 2D channel geometry, with length $L = 80$ mm and width $W = 15$ mm. Finally, three-dimensional geometries are introduced to verify that the mechanisms underlying diodicity persist in higher dimensions and to explore additional effects specific to 3D flows. In this case, the channel is a tubular conduit with diameter $D = 20$ mm and length ranging from $L = 35$ to 80 mm.

2.2. Governing Equations

All simulations were performed in the COMSOL Multiphysics (v6.3; COMSOL AB, Stockholm, Sweden) environment [24]. For the rigid configurations, the geometry is fixed and the flow is governed by the incompressible Navier–Stokes equations:

$$\rho \left(\frac{\partial \mathbf{v}}{\partial t} + \mathbf{v} \cdot \nabla \mathbf{v} \right) = -\nabla p + \mu \nabla^2 \mathbf{v}, \quad \nabla \cdot \mathbf{v} = 0,$$

where $\mathbf{v}$ is the velocity field and p is the pressure. Water was used as the working fluid, with density $\rho = 10^3$ kg/m³ and dynamic viscosity $\mu = 10^{-3}$ Pa · s.

When the obstacle is free to deform, the problem becomes fully coupled with a solid domain governed by momentum balance:

$$\nabla \cdot \boldsymbol{\sigma} + \mathbf{b} = \rho_s \ddot{\mathbf{u}},$$

where $\mathbf{u}$ is the displacement field, $\boldsymbol{\sigma}$ is the Cauchy stress tensor, derived from a hyperelastic Neo-Hookean constitutive law, and ρ_s is the density of the solid domain. Fluid and solid domains share a common interface where continuity is imposed:

$$\mathbf{v} = \dot{\mathbf{u}}, \quad \boldsymbol{\sigma}_f \mathbf{n} = \boldsymbol{\sigma}_s \mathbf{n}.$$

with $\boldsymbol{\sigma}_f$ and $\boldsymbol{\sigma}_s$ representing the fluid and solid stresses, and $\mathbf{n}$ the interface normal.

To facilitate comparison across geometries and flow conditions, and with the existing literature, we characterize the flow regime using the Reynolds number,

$$\text{Re} = \frac{\rho U L_c}{\mu}, \quad (3)$$

where U is the imposed inlet velocity and L_c is a characteristic length scale of the channel. In the 2D configurations, L_c is taken as the channel width, while in 3D geometries, it is defined as the hydraulic diameter. The ranges of Reynolds numbers explored in each configuration are reported together with the corresponding results.

2.3. Study Setup

The boundary conditions follow standard configurations for fluid dynamics problems: a prescribed inlet condition, specifying either a constant uniform velocity U_{in} or a non-zero relative pressure $P > 0$, zero relative pressure at the outlet, and no-slip on all rigid walls. In the deformable cases, the obstacle base has zero prescribed displacement, while the remaining solid boundaries are free to deform under the coupled fluid–structure interaction.

The mesh consists of triangular elements in 2D and of tetrahedral elements in 3D, with rectangular or prismatic boundary-layer elements near the walls, generated by COMSOL Multiphysics (v6.3), using the “physics controlled” meshing option. Mesh motion followed the structural deformation through an ALE formulation, ensuring stable grid quality and solver convergence. Mesh-convergence tests were performed to ensure that the results are independent of the element size. Representative 2D results for a specific test case are reported in Table 1. There, for meshes with number of elements $\geq 1.2 \times 10^4$, the relative deviation on the pressure drop ΔP with respect to the finest mesh remains below 1%. Unless otherwise stated, all 2D simulations reported in this work were performed with $\sim 1.2 \times 10^4$ elements.

The equations were solved with a fully coupled time-dependent solver. Each simulation was run until a quasi-steady regime was reached: either a steady state with negligible temporal variations in pressure drop and flow rate, or a periodic limit cycle. In the latter case, pressure drop and flow rate were post-processed as time-averaged quantities over several oscillation periods.

Table 1. Representative 2D mesh-convergence study for the rigid obstacle at $\alpha = 50^\circ$, monitoring the pressure drop ΔP . The finest mesh (number of elements = 617,898) is used as a reference to compute the relative error.

#	Elements	ΔP	Rel. Err.
1	2506	0.91976	3.17%
2	4734	0.92852	2.25%
3	7006	0.93617	1.44%
4	12,	0.94484	0.53%
5	28,130	0.94602	0.40%
6	66,728	0.94830	0.16%
7	118,952	0.94885	0.11%
8	617,898	0.94986	0.00%

3. Results

We first analyze a simple 2D configuration, used as a baseline to define the forward and backward pressure drops, ΔP^+ and ΔP^- . Figure 4 shows the reference geometry and the corresponding pressure distributions in the two flow directions.

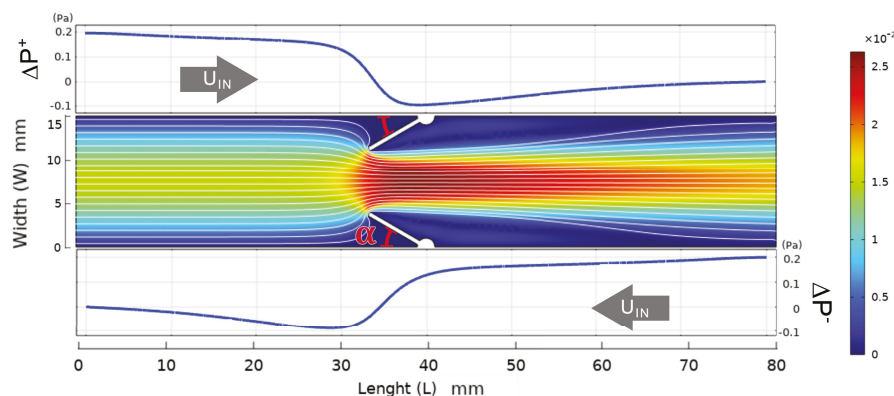


Figure 4. Reference 2D channel used for both rigid and deformable simulations. The obstacle is placed at mid-length and rotated by an angle α . The plots show the pressure along the channel centerline for forward (ΔP^+ , **top**) and reverse (ΔP^- , **bottom**) flow, illustrating the asymmetric pressure distribution and the partial pressure recovery downstream of the obstacle. Color legend indicates velocity magnitude.

3.1. Two-Dimensional Rigid Channel

We vary the obstacle angle $\alpha \in [0^\circ, 90^\circ]$ and the inlet velocity $U_{in} \in [0.01, 0.1]$ m/s. These ranges were selected based on prior parametric tests to cover the regime where diodicity becomes appreciable. Figure 5 shows representative flow fields for $\alpha = 30^\circ$ and the plot of diodicity as a function of α .

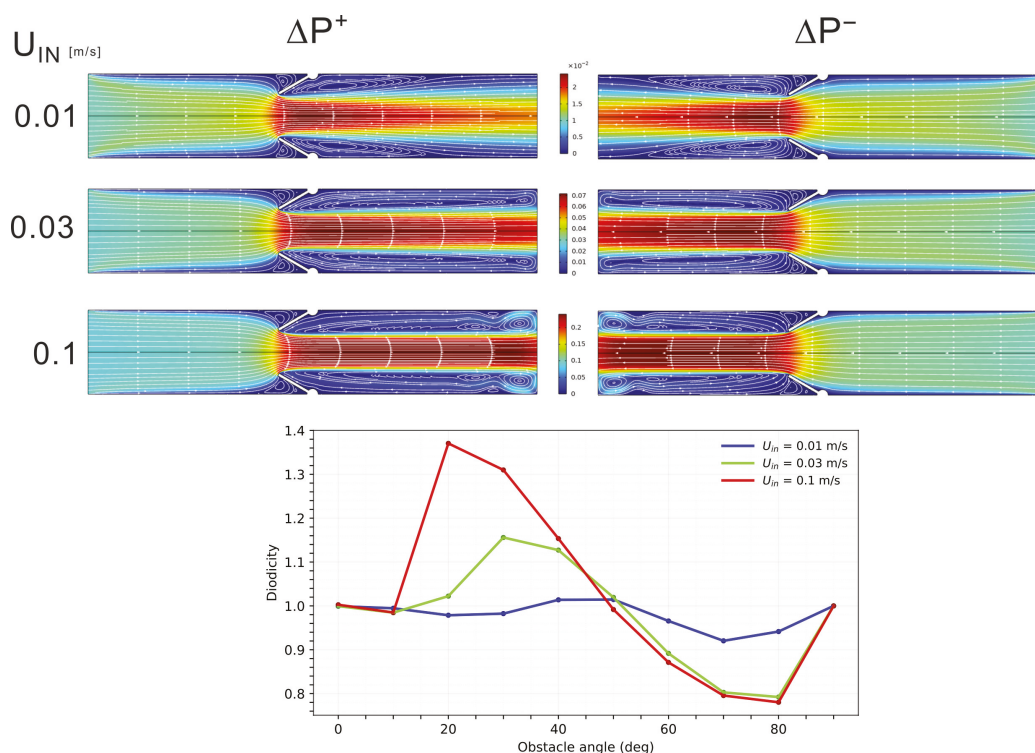


Figure 5. (Top) Velocity fields for the 2D rigid channel with $\alpha = 30^\circ$ in the forward (ΔP^+) and backward (ΔP^-) configurations at different inlet velocities. The color legend is shared across rows and indicates the velocity magnitude. (Bottom) Diodicity curves $D(\alpha)$ for increasing inlet velocities U_{in} , showing higher peak values at larger U_{in} .

The diodicity trends can be interpreted by inspecting the recirculation zones in the two directions. Stronger recirculation implies larger viscous and inertial losses: when recirculation is stronger in the backward case, $\Delta P^- > \Delta P^+$ and $D < 1$. The opposite

configuration yields $\Delta P^+ > \Delta P^-$ and $D > 1$. Thus, even a single rigid obstacle is sufficient to produce purely geometric, direction-dependent hydraulic resistance.

3.2. Two-Dimensional Deformable Channel

The same reference geometry is then considered with the obstacle allowed to deform. We vary the obstacle orientation $\alpha \in [0^\circ, 90^\circ]$ and the inlet velocity $U_{in} \in [10^{-3}, 10^{-2}]$ m/s, while keeping the elastic modulus fixed at $E = 10^4$ Pa. These values and ranges were selected based on prior parametric tests to cover the regime where diodicity becomes appreciable. Representative deformed configurations and the corresponding curves showing diodicity as a function of U_{in} and at fixed α are shown in Figure 6.

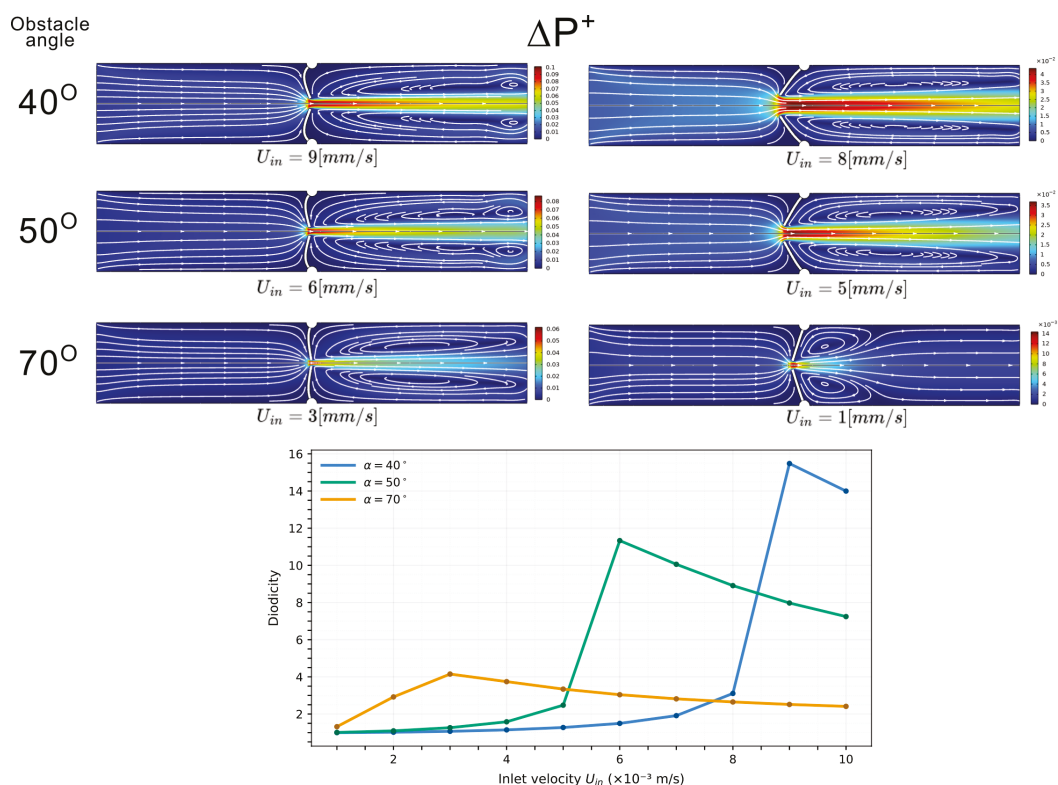


Figure 6. (Top) Two-dimensional deformable case, velocity field, and streamlines for forward flow (ΔP^+) at fixed elastic modulus $E = 10^4$ Pa. Each row corresponds to a different obstacle angle α , and each column to a different inlet velocity U_{in} , leading to different deformation levels and effective apertures. Color bars indicate the velocity magnitude. (Bottom) Plot of diodicity as a function of U_{in} and at fixed α , highlighting a peak value at a specific combination of E , U_{in} , and α where the channel is most obstructed.

The diodicity response reflects how the obstacle deforms. In the forward configuration, the obstacle bends into the channel and sharply reduces the cross-section; in the backward case, it bends away and opens the passage. Consequently, a larger pressure drop is required in the forward direction to sustain the same flow rate. A “sweet spot” appears when the deformation produces near-maximal occlusion, causing a sharp peak in diodicity. Small changes in inlet velocity can therefore trigger large reconfigurations of the obstacle and, as a result, strong variations in diodicity.

3.3. Annuli Model

To separate the roles of elasticity and geometry, we introduce a simplified model that mimics the effect of deformation with a rigid obstacle whose opening matches the minimum opening h of the deformed geometry between obstacles tips (Figure 7).

We simulate the 2D deformable case at fixed inlet velocity $U_{in} = 0.01$ m/s and obstacle angle $\alpha = 30^\circ$, varying the elastic modulus in the range $E \in [0.5, 50]$ kPa to generate different deformed shapes. This range was selected based on prior parametric tests to span the regime where the deformation-induced minimum aperture (and hence diodicity) becomes appreciable. These shapes are then used to define the opening of the rigid barrier of the equivalent model.

The diodicity values in the deformable and equivalent rigid cases almost overlap, indicating that the dominant contribution does not arise from elasticity itself, but from the geometric reconfiguration generated by deformation.

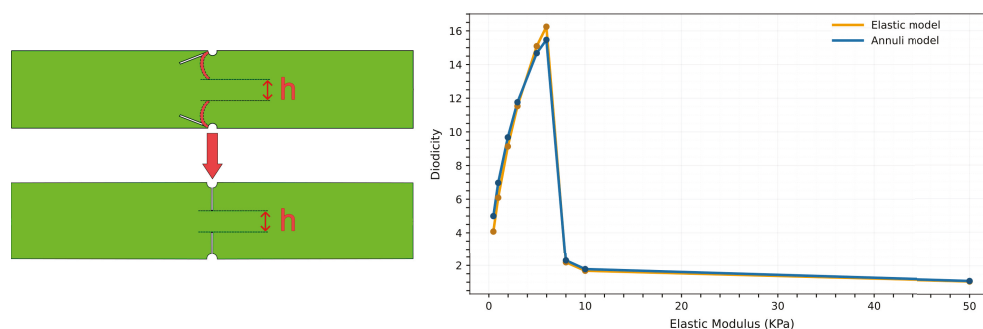


Figure 7. Equivalent annuli model. **(Left)** The original deformed configuration is replaced by a rigid barrier with the same minimum opening h . **(Right)** Comparison between diodicity values in the deformable and equivalent rigid cases, showing minor differences and indicating that geometry is the main contributor to the asymmetric effect.

3.4. Complete Blockage Regime

We now consider a bioinspired configuration, taken from the lymphatic system and shown in Figure 8, in which full blockage may occur and the diodicity definition (1) becomes impractical. The model consists of two deformable, leaflet-like obstacles forming a nearly closed channel, with the same wall geometry as in the previous 2D configuration. The elastic modulus is fixed at $E = 10^4$ Pa, chosen to be consistent with pressure levels comparable with those observed in murine lymphatic vessels [1]. When a pressure difference is applied in the forward direction (ΔP^+), the two leaflets are pushed against each other, closing the channel. In the reverse direction (ΔP^-), the flow separates the leaflets and opens the passage.

As noted in Section 2, we characterize this regime by imposing a pressure difference $\Delta P \in [0, 1]$ Pa and measuring the resulting flow rates $Q(\Delta P^\pm)$ in both directions. This range is consistent with values used in modeling physiological lymphatic valves (see [1]), where applied pressure differences are in the order of a few dyne/cm² (e.g., $\Delta P \approx 6$ dyne ≈ 0.6 Pa). Since the simulations are performed in 2D, the volumetric flow rate Q is obtained by assuming, for simplicity, a unit out-of-plane thickness of 1 mm:

$$Q = q \cdot 1 \text{ mm}, \quad (4)$$

where q is the computed planar flux.

In this framework, a symmetric channel would yield comparable flow rates in the two directions for the same pressure drop, $Q(\Delta P^+) \approx Q(\Delta P^-)$. In contrast, a valve-like response is characterized by $Q(\Delta P^+) \approx 0$ and $Q(\Delta P^-) > 0$ for the same ΔP . Our simulations fall in this latter regime, indicating nearly one-way transport.

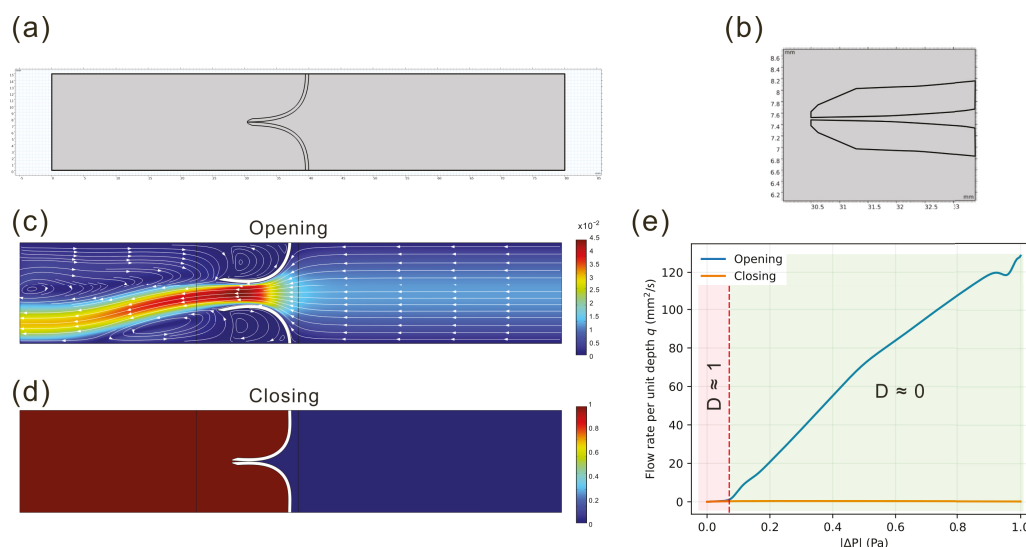


Figure 8. (a–d) Geometry and elastic response of the leaflet valve. (a) Reference configuration with two opposing flexible leaflets; (b) zoomed-in view of the valve region showing the small initial gap between the leaflets. (c) Velocity magnitude and streamlines in the opening case: the pressure drop separates the leaflets, and a jet crosses the gap and relaxes downstream towards a uniform profile; its slight lateral bending is a symmetry-breaking instability that does not affect the integrated flux. (d) Pressure field in the closing case: the reversed pressure pushes the leaflets into contact, forming a barrier and suppressing transport. (e) Quasi-steady flow–pressure relation $q(\Delta P^\pm)$ in both directions. Beyond a small occlusion threshold (red dashed line), the closing branch (ΔP^+) remains negligible, while the opening branch (ΔP^-) grows almost linearly with $\Delta P^\pm$. The shaded bands mark regimes of diodicity D : a nearly symmetric response ($D \approx 1$) at low load and a complete-blockage regime ($D \approx 0$) where the diodic behavior saturates and the valve acts as an almost one-way conduit.

3.5. Three-Dimensional Channel Results

In this section, we investigate the flow behavior in three-dimensional channels containing rigid and deformable obstacles.

3.5.1. Rigid Cone-Shaped Obstacle

We first analyzed how the angle of the obstacle influences the diodicity of a channel equipped with a rigid cone-shaped obstacle. This 3D geometry was constructed by revolving the profile of the corresponding 2D channel about its axis. In all simulations, a constant inlet velocity of 0.1 m/s was imposed to match the 2D boundary conditions and enable direct comparisons across models. Figure 9a presents the flow streamlines in both directions for the 3D channel with a cone angle of $\alpha = 20^\circ$. Figure 9b shows the diodicity as a function of obstacle angle for both the 3D and 2D simulations. Although there are discrepancies at some angles between the two sets of results, the overall trend from the 2D simulations aligns well with the 3D data.

3.5.2. Rigid and Deformable Incomplete Cone-Shaped Obstacles

To explore the mechanism responsible for the strong diodicity observed in channels with 2D deformable obstacles, we developed a 3D model consisting of an incomplete cone made of six leaflets (Figure 10a). The geometry was optimized to avoid buckling and contact between the leaflets during deformation. The obstacle's initial opening angle was set to $\alpha = 40^\circ$, and its elastic modulus was set to $E = 1$ kPa. As shown in Figure 10c, the channels with these incomplete cone obstacles exhibit a significant increase in diodicity in the deformable case compared with the rigid one, particularly when the opening area decreases markedly for forward (ΔP^+) flow (Figure 10b) under an inlet velocity of 0.012 m/s.

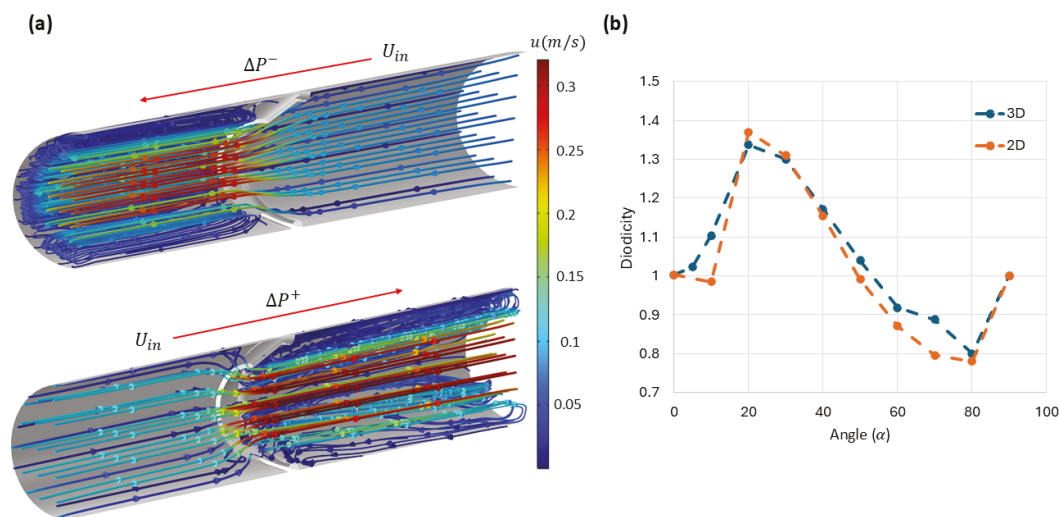


Figure 9. Fluid flow through a 3D channel with a rigid cone-shaped obstruction at a fixed inlet velocity of 0.1 m/s. (a) Streamlines for flow in opposite directions; the color bar indicates the magnitude of the velocity field. For clarity, only half of the channel is shown. (b) Diodicity values calculated for various obstacle angles, compared with the 2D simulations in Figure 5.

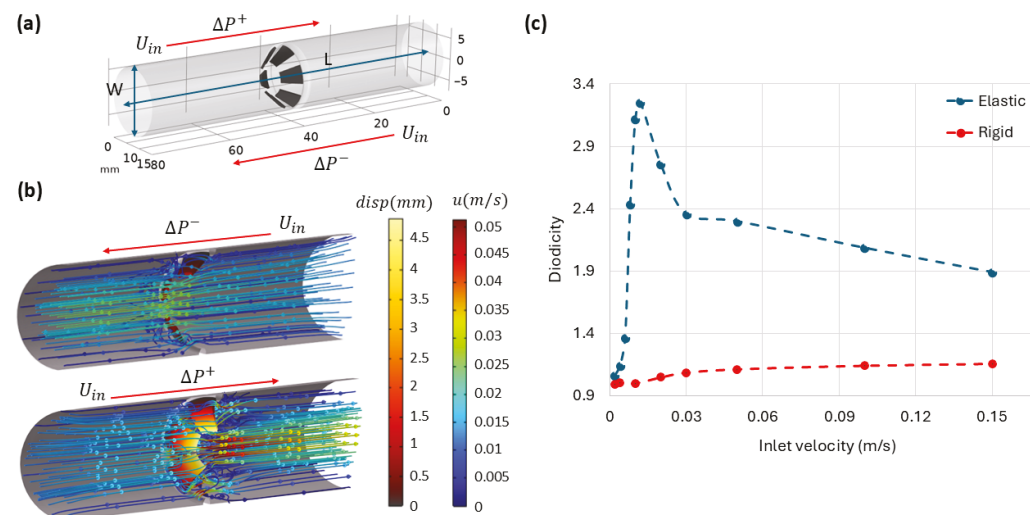


Figure 10. Fluid flow through 3D channels with rigid and deformable incomplete cone-shaped obstruction for a fixed angle and various inlet velocities. (a) Geometry of the 3D channel with an incomplete cone-shaped obstacle composed of six leaflets. The pressure drops for forward and backward flow are denoted by ΔP^+ and ΔP^- , respectively. (b) Streamlines for flow in both directions through the channel with a deformable obstacle, along with the corresponding deformation under fluid force. The color bars indicate the displacement magnitude ($disp$) of the obstacle and the magnitude of the fluid velocity (u). For clarity, only half of the channel is shown. (c) Computed diodicity for channels with both elastic ($E = 1$ kPa) and rigid obstacles over different inlet velocities.

It is worth noting that the maximum diodicity obtained in this 3D configuration is around 3.3, which is notably smaller than the maximum value observed in 2D simulations. This difference arises because, in the 3D simulations, finite gaps always remain between the leaflets, allowing some fluid to pass through even when the obstacle deforms. Nevertheless, these results confirm that obstacle elasticity and the reduction in the effective opening area are key factors in enhancing diodicity. These observations are fully consistent with the mechanisms identified in the 2D simulations.

3.5.3. Shark-Intestine-Inspired Obstacles

Lastly, we examined flow asymmetry in channels with rigid spiral obstacles inspired by the structure of the shark intestine. The geometries were constructed based on the experimental study in [2]. The inlet velocity was kept constant at 0.38 m/s. Figure 11a shows the flow streamlines in both directions, highlighting the inherent asymmetry in the fluid motion. We then performed a parametric analysis of the flow in both the top-to-bottom and bottom-to-top directions, varying the inner radius of the spiral obstacle as well as the number of turns. Figure 11b,c, compare the computed numerical diodicities for various inner radii and numbers of turns, with the experimental results reported in [2]. As shown, the numerical simulations confirm the presence of flow asymmetry in channels with rigid spiral obstacles, and the overall trends align reasonably well with the experimental observations.

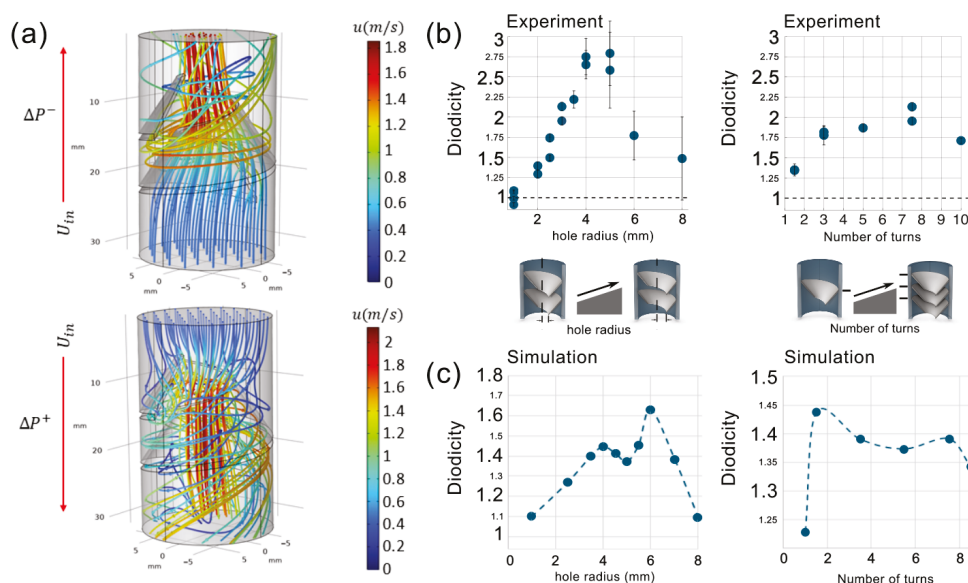


Figure 11. Fluid flow through 3D channels with a rigid spiral obstacle inspired by the shark intestine. (a) Streamline patterns for flow in both directions. Numerical and experimental [2] diodicity values for spiral obstacles with (b) various inner hole radii and (c) different numbers of turns.

However, some discrepancies in the predicted diodicity values can be observed. Figure 12 shows the absolute percentage error in diodicity for various hole radii for the 3D channel with a spiral obstacle. We attribute the larger error values in the central region of the diagram to mismatches associated with uncertainties in the experimental setup. In fact, we are able to see diodicity values approaching 2 and beyond only by invoking the deformability of the spiral obstacle (see Figure 13), while for the perfectly rigid case, we could not exceed the value of 1.65. The larger errors correspond precisely to the regime in which we underestimate the diodicity with our perfectly rigid obstacle. Even small deformability would shift the predictions to higher values, reducing the error with respect to the experiment.

We also performed a series of simulations to investigate how obstacle deformability affects diodicity in channels with spiral obstructions. In these simulations, the number of turns was fixed at 1.5, the pitch distance at 7.5 mm, and the inner radius at 3 mm, while the inlet velocity was varied. The elastic modulus of the obstacle was set to $E = 1$ MPa. Figure 13 shows that the elastic behavior of the obstacle increases the diodicity compared with the rigid case as the inlet velocity is increased. At higher inlet velocities, however, the simulations experienced convergence challenges due to contact interactions and structural instabilities within the deformable spiral. As a result, diodicity values could not be obtained

in this regime using the current modeling framework. Addressing these limitations will require more advanced numerical techniques and is left for future work.

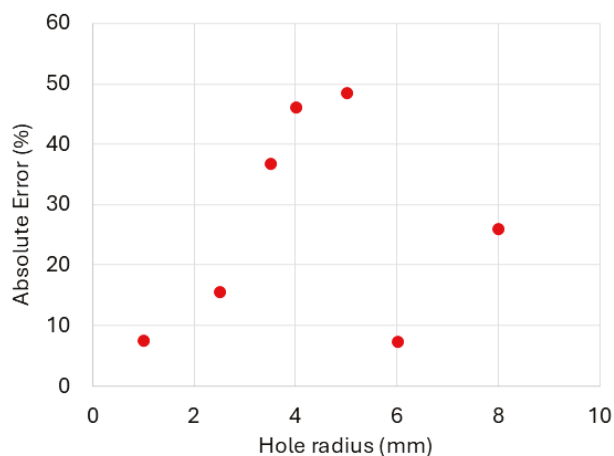


Figure 12. Absolute percentage error in diodicity for 3D channel with spiral obstacle obtained from numerical simulations relative to experimental measurements for different inner hole radii.

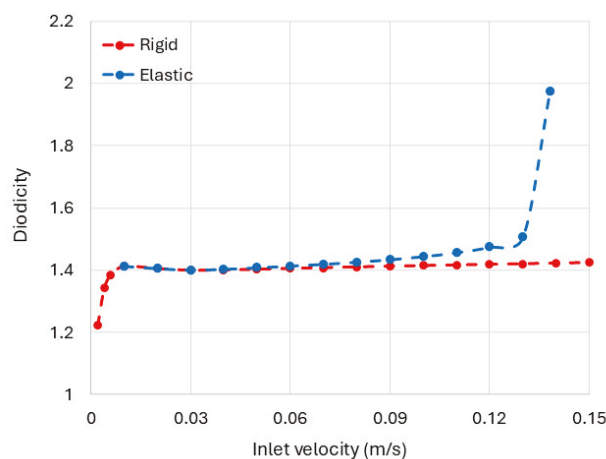


Figure 13. Computed diodicity for channels with both elastic ($E = 1$ MPa) and rigid spiral shark-intestine-inspired obstacles over different inlet velocities. In these simulations, the number of turns is 1.5, the inner radius is 3 mm, and pitch distance is 7.5 mm.

4. Discussion

The configurations analyzed in this work show that hydraulic asymmetry in tubular channels can be interpreted within a single geometric framework. Hydraulic asymmetry emerges when reversing the flow direction reorganizes the topology of streamlines. This cannot occur in the case of flows in undeformable domains with fore-aft symmetry, or in the limit of vanishing Reynolds numbers, because of symmetries in the governing equations. In the presence of elastic obstacles, the flow reshapes the channel's effective aperture in a direction-dependent manner. The examples discussed in the Introduction can thus be revisited under this unified lens.

In the 2D rigid case, asymmetry arises from geometric steering alone. Reversing the flow direction reorganizes streamline topology, as well as inertial and viscous losses. Then different pressure drops, ΔP^+ and ΔP^- , are required to maintain the same mean flow rate. This mechanism is well documented in the literature on Tesla-type valves through detailed numerical and experimental investigation. We use these results as a quantitative benchmark for passive fluidic diodes. Here, however, we consider a markedly different

setting: a single-stage channel with an obstacle inspired by biological architectures. Despite the absence of design optimization, our results show that such geometric bias already produces a measurable diodicity, without moving parts or material nonlinearity. In our simulations, inlet velocities of $U_{\text{in}} = 0.01\text{--}0.1$ m/s yield peak diodicity values in the range $D \simeq 1.01\text{--}1.37$, corresponding to Reynolds numbers of $Re \simeq 150\text{--}1500$. Although smaller than those reported for optimized multistage Tesla valves ($D \sim 1.8\text{--}2.3$ at comparable Reynolds numbers [15]), these values demonstrate that geometric steering alone can already produce a significant directional bias, in geometries much simpler than Tesla's.

In the 2D deformable channel, forward and backward flow bend the obstacle toward or away from the main stream path, narrowing or reopening the passage. Replacing the deformed obstacle with an equivalent rigid barrier with the same minimum opening h leaves the diodicity almost unchanged. This shows that the key quantity is the aperture selected under load. This single parameter captures the competition between hydrodynamic forces and elastic resistance, which is strongly nonlinear. As a consequence, small variations in inlet velocity can trigger abrupt changes in the selected aperture, producing sharp peaks in the diodicity curves. In our simulations, the peak diodicity value reaches $D \simeq 15.5$ at a Reynolds number of $Re \simeq 135$. By comparison, numerical studies on Tesla-type valves report peak values around $D \simeq 4.4$ at $Re \sim 300$, and lower diodicities at Reynolds numbers comparable to those explored here [15].

The bioinspired valve configuration extends this mechanism to a regime in which the passage is almost completely closed in one direction while remaining open in the other. The same principle underlies physiological valves in the heart and lymphatic system, where backflow is suppressed in an analogous manner. Our valve-like simulations reproduce this behavior.

Three-dimensional simulations confirm that hydraulic asymmetry persists in fully three-dimensional geometries. For incomplete conical obstacles, the diodicity values attainable in 3D are lower than their 2D counterparts, as the maximum achievable asymmetry is limited by finite gaps. In shark-intestine-inspired obstacles, our 3D rigid simulations capture the experimentally observed trends as the inner radius and the number of turns vary. Introducing deformability further enhances diodicity, although numerical issues associated with contact restrict the range of velocities that we could explore. Previous numerical studies on Tesla valves have shown that turbulence models can improve quantitative agreement with experiments at moderate Reynolds numbers ($Re \sim 500\text{--}2000$) [15,25]. At higher Reynolds numbers ($Re \sim 7600$), our preliminary tests using a RANS $k\text{--}\epsilon$ model in our more complex spiral geometry do not lead to significant changes in the computed values of diodicity. A detailed turbulence-model sensitivity analysis is left for future work. Our analysis suggests that the 3D design based on an internal helical obstacle, loosely inspired by the anatomy of the shark intestine, does not reproduce one of the key mechanisms that generate hydraulic asymmetry in classical 2D Tesla valves. This is illustrated in Figure 14.

Indeed, in the Tesla valve case, the conflict between primary and secondary streams arising in the backward flow direction (black and red streamlines, respectively) leads to larger hydrodynamic losses than in the forward flow direction (blue streamlines) and, hence, to potentially large values of the diodicity. By contrast, in the case of the 3D shark-intestine-inspired helical structure, the primary and secondary streams in the backward direction have components along the tube axis with the same sign. Thus, the secondary stream induces smaller hydrodynamic disturbance of the primary stream, and lower hydrodynamic losses with respect to the 2D Tesla scenario. It is left to future investigations to explore whether by fine tuning the 3D obstacle geometry (e.g., by considering different ratios of pitch, external radius, or internal hole radius, or by reproducing the shark anatomy more faithfully) or, alternatively, by exploring 3D geometries departing more drastically

from the shapes bio-inspired by the shark intestine anatomy, one may arrive at diodicity values larger than the ones we have found in our study.

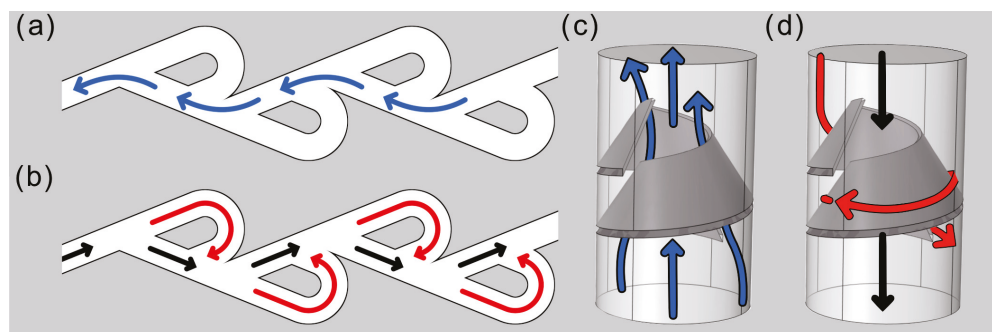


Figure 14. Conceptual comparison between the Tesla-valve principle and the shark-intestine-inspired geometry. Schematic flow pathways highlight how geometric features generate secondary streams (red) that interact with the primary through-flow (black). Flow in the opposite direction (blue) is mainly undisturbed by secondary flows. Tesla-valve configuration (a,b) promotes pronounced secondary recirculation. Shark-intestine-inspired geometry (c,d) yields weaker disturbance of primary flow by secondary streams.

From a design viewpoint, the primary control parameter is the effective minimum opening selected under load. For a given operating range of flow speed U and obstacle angle α , our models (Section 3.3) identify stiffness values E that yield the smallest selected aperture and therefore maximize hydraulic asymmetry. Conversely (Section 3.2), in deformable-obstacle simulations at fixed E , varying U and α controls the selected aperture and determines whether the response remains weak or becomes strongly asymmetric. The triplet (U, α, E) therefore defines a compact design space that links driving, geometry, and stiffness to the selected minimum opening. The same parameter space applies to valve-like operation, where performance is expressed in terms of flow rate rather than hydraulic asymmetry.

This framework can be used as a practical design map. In soft-valve concepts, we can tune E so that physiological pressure loads induce markedly different deformations (and thus different minimum openings) in forward versus reverse flow. In rigid devices, we can select (U, α) in the regimes where directional recirculation is most pronounced to obtain robust baseline asymmetry. Overall, this approach puts non-occluding and valve-like architectures on the same footing and provides practical guidelines to design passive soft devices for directional flow control.

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Article

Mechanical Characterization of Stick Insect Tarsal Attachment Fluid Using Atomic Force Microscopy (AFM)

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Abstract

Most insects secrete special fluids from their tarsal pads which are essential for the function of their attachment systems. Previous studies investigated several physical and chemical characteristics of this pad fluid in different insect species. However, there is not much known about the mechanical properties of fluid from smooth adhesive pads. In this study, we used the stress–relaxation nanoindentation method to examine the viscoelastic properties of pad fluid from *Sungaya aeta*. Force–displacement and stress–relaxation curves on single fluid droplets were recorded with an atomic force microscope (AFM) and analyzed using Johnson–Kendall–Roberts (JKR) and generalized Maxwell models for determination of effective elastic modulus (E), work of adhesion ($\Delta\gamma$) and dynamic viscosity (η). In addition, we used white light interferometry (WLI) to measure the maximal height of freshly acquired droplets. Our results revealed three different categories of droplets, which we named “almost inviscid”, “viscous” and “rigid”. They are presumably determined at the moment of secretion and retain their characteristics even for several days. The observed mechanical properties suggest a non-uniform composition of different droplets. These findings provide a basis for advancing our understanding about the requirements for adaptive adhesion-mediating fluids and, hence, aid in advancing technical solutions for soft or liquid temporal adhesives and gripping devices.

Keywords: wet attachment; tarsal secretion; force measurements; nanoindentation; effective modulus; viscosity

1. Introduction

Insects have lived and evolved on our planet for 400 million years. They are commonly known to show the largest diversity of all taxa within the animal kingdom [1]. One reason often used to explain this success is their ability of reversible attachment to almost any surface and, consequently, to locomote on them [2]. There are two general principles of tarsal attachment systems [3] that evolved in several groups of insects independently [4,5]. The first type are hairy systems, which are composed of a vast number of tiny setae (hairs). They can be found in flies and beetles, for example, but also in other groups of animals, like geckos and spiders [6]. The other type, smooth pads, does not involve any setae, but instead consists of highly flexible and soft cuticle [7], in contrast to the sclerotized cuticle of the exoskeleton. This type can be found, for example, in grasshoppers and bugs [3,8]. However, both types of tarsal attachment systems increase adhesion by adapting to the substrate’s profile to maximize the real contact area [9]. Furthermore, insects use an

additional mechanism to enhance their attachment, by secreting a small layer of secretory fluid into the contact zone.

The phenomenon of wet attachment has gained plenty of attention from scientists during the last decades, and there have been lots of studies that examined the complex interaction of pads, substrate and fluid in various insect species [10–12]. The results of these studies show that a loss of fluid has strong effects on insect attachment. In addition, some studies focused more on the mechanical properties of the fluid itself, e.g., using rheology [13,14] and evaporation rate measurements [15]. Furthermore, the fluid plays a role in self-cleaning of pads, as it can fill the gaps between contaminating particles [16], and it can also affect the surface chemistry and hydrophobicity [17]. Other studies examined the chemical composition of fluid [18–20], and some even tested bioinspired artificial synthetic emulsions [21]. However, the influence of the adhesion fluid's properties on the attachment abilities is not yet understood in detail [22].

Although biological surfaces are complex in general, there have been several studies which examined the attachment pads of several animal species that involve fluids, including insects and also some arachnids [10,23,24]. Referring to these studies, the attachment fluid mainly contributes to the surface conformity of the attachment pad with the substrate, enhancing the contact area on rough surfaces, but might also have further effects [11]. Another study postulated that systematic changes in the fluid's properties might not only help to optimize attachment but could also compensate for the increasing body mass of growing stick insects. However, they found that the fluid's properties were independent from body size [25]. Nevertheless, the mechanical properties of the solid pad material are determined by the cuticle ultrastructure. On the other hand, the properties of the fluid might be variable, based on chemical or physical composition, which probably allows further adaptation during the contact period. One previous study already showed that there is a difference in fluid viscosity in the hairy pads of beetles and flies, as well as a correlation between fluid viscosity and contact formation time during attachment [14]. This indicates that, for both examined species, there is a specific optimal value of fluid viscosity for attachment.

However, to the best of our knowledge, there have been no studies yet that directly measured the viscoelastic properties of the attachment fluid from smooth adhesive pads, as the secreted amounts of fluid from these pads were too small to use conventional methods of analysis. Despite the fact that there has been no direct analysis yet, our previous observations led to the assumption that fluid from smooth pads differs in its physical and chemical properties when compared to hairy pads. Furthermore, investigation of the frozen tarsal secretion of the stick insect *Medauroidea extradentata* (Brunner, 1907), which possesses smooth adhesive devices, revealed the presence of morphologically distinct components in the footprints of this species. It was hypothesized that these morphological differences and the range of evaporation rates represented in different fluid droplets in the secretion are a result of dissimilar physicochemical properties within the secretion [26].

In this study, we used atomic force microscopy (AFM) to examine the viscoelastic properties of tarsal fluid from the stick insect species *Sungaya aeta* Hennemann 2023, as a representative of the insects that have smooth pads. With the atomic force microscope, we were able to perform a precise analysis of local forces on the nanometer scale, which allowed for the investigation of single fluid droplets from freshly obtained footprints without the need to collect larger amounts of fluid. For this investigation, we recorded force–distance curves on single fluid droplets at different time points. Afterwards, we used the Johnson–Kendall–Roberts (JKR) model to calculate the effective modulus (E) and the surface energy from these curves, and we also investigated the dynamic viscosity (η). The aim of this study was to collect qualitative results about the viscoelastic properties

of smooth pad fluids, which can be compared to our assumptions, as well as to previous findings on hairy pads.

2. Materials and Methods

2.1. Footprint Acquisition

For all experiments, only adult female individuals of stick insect species *S. aeta* (Figure 1A), originating from the lab culture of the Department of Functional Morphology and Biomechanics (Kiel University, Germany), were used. Previous studies on adhesive systems of stick insects mentioning *Sungaya inexpectata* ZOMPRO, 1996 most certainly worked on the same species (see Grote et al. 2024) [27]. The insects were kept at a natural day/night cycle and were fed with leaves of blackberry and hazel ad libitum. All individuals showed no injuries or signs of reduced health during the experiments. Footprints for force measurements were taken from random tarsi of two individuals. Therefore, one tarsus (Figure 1B) was positioned on the marked center of a glass slide, and the insect's body was manually moved to stimulate the attachment pads for approximately 60 s. After removing the tarsus, the resulting footprint was checked first visually and afterwards in the light microscope (Figure 1C). Only footprints showing fluid droplets of more than 10 μm diameter were used for measurements. Fluid from both types of pads, arolium and euplantulae (Figure 1B), was considered.

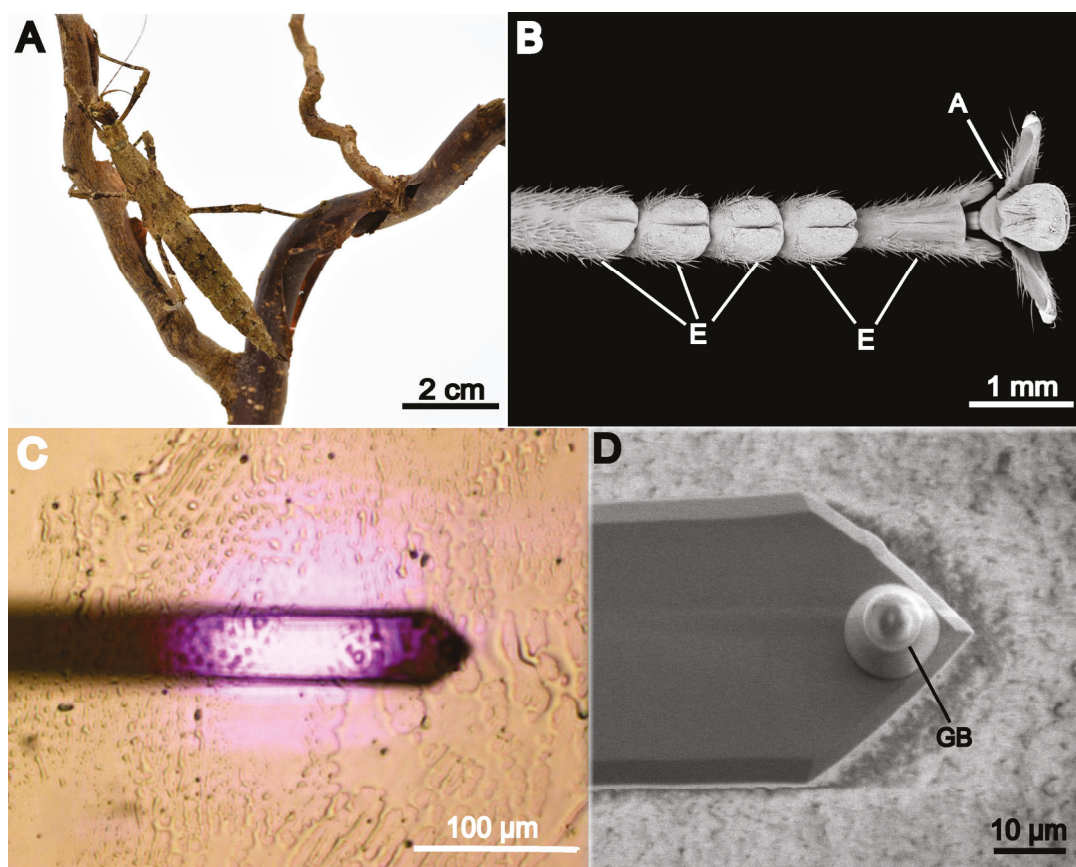


Figure 1. Animals and structures studied. (A) *Sungaya aeta* adult female. (B) Scanning electron microscopy (SEM) picture of tarsus from *S. aeta*, showing the attachment pads euplantulae (E) and arolium (A). (C) Inverse light microscopy (LM) observation of a footprint during AFM force measurements, observed from below, through the glass slide, showing the cantilever approaching the tarsal fluid droplets. (D) SEM picture of another cantilever tip with attached glass bead (GB) as an example.

Footprints used for white light interferometry (WLI) measurements were taken from five different individuals. For this experiment, four different treatments were performed during footprint acquisition. In the first treatment, the animal was placed on the glass slide and left unaffected for up to 6 min before removing it (1). In the second treatment, the animal was held in place with its tarsus in contact to the slide, which caused the animal to push its tarsus actively against the slide (2). In the third and fourth treatment, we gently moved the animal with our hand across the slide, with its tarsi in contact either in pushing (3) or pulling (4) direction. During footprint acquisition, sequence of animal, tarsi and treatment were randomized. Again, fluid from both types of pads, arolium and euplantulae (Figure 1B), was considered.

2.2. White Light Interferometry

Height measurements of freshly acquired fluid droplets were carried out with the white light interferometer (Zygo NewView 6000, Zygo Corporation, Middlefield, CT, USA) in combination with the software MetroPro (version 8.3.5, Zygo Corporation, Middlefield, CT, USA), using the $5\times$ and $20\times$ objectives. Height profiles were taken to measure the maximal height of the fluid droplets in each selected region.

2.3. Cantilever Preparation

For fluid examination, a tipless cantilever with a spring constant of 0.1046 N/m (CSC38/tipless/No Al, 0.03–0.09 N/m, μ masch, Sofia, Bulgaria) was used. The spring constant was obtained using the well-established thermal noise method. The cantilever tip was supplemented by the glass bead (Borosilicate Glass Microspheres, $7.9 \pm 0.8 \mu\text{m}$ diameter, Duke Scientific Corporation, Palo Alto, CA, USA) attached to it by employing epoxy glue (UHU Plus Schnellfest, 2-k-epoxy-glue, Uhu GmbH & CoKG, Bühl, Germany). The bead's actual size was measured using LM images of the attached bead and LM images of a scaling microscopy slide as a reference, giving a radius of $5.092 \mu\text{m}$. Prior to the measurements, the sensitivity of the cantilever was calibrated 10 times at each of 16 different positions on a glass slide, using a setpoint of 5 V. Then, the mean value acquired from this calibration was used. In addition, we took scanning electron microscope images of a prepared cantilever using a Hitachi S 4800 scanning electron microscope (Hitachi High Technology, Tokyo, Japan) to visualize the true shape of the glass bead (Figure 1D).

2.4. Force Measurements

All measurements were carried out with the atomic force microscope (AFM) (NanoWizard, A0016, Bruker Nano GmbH, Berlin, Germany), using the NanoWizard Control Software (version 4, Bruker Nano GmbH, Berlin, Germany). For visual control, the AFM was combined with an inverse light microscope (Axiovert 135, Zeiss, Oberkochen, Germany), equipped with a camera (Imaging Source, DFK 31AF03, Bremen, Germany). Visual observation was carried out with $20\times$ objective. During each measurement, the cantilever indented a droplet and was then kept at constant height for 3 s before retraction. We chose a setpoint force of 10 nN, a Z-range of $10 \mu\text{m}$ and an approach speed of $10 \mu\text{m/s}$. The AFM head motion in the z-direction was performed in a closed-loop operation.

In detail, each measurement started with the cantilever approaching the droplet surface. The cantilever motion continued until the setpoint force was reached. During the following delay phase, the cantilever deflected passively (no AFM head motion) because of the deformation of the droplet below. At the end of the delay phase, the cantilever was retracted, undergoing a strong pulling force because of its adhesion to the droplet, until the contact broke and the cantilever jumped back to its equilibrium position (Figure 2). After every single measurement on a droplet, the eventual fluid sticking to the cantilever was stripped off by moving the cantilever along the glass slide. This procedure was always

followed by a control glass indentation prior to the next measurement. As curves recorded with a clean cantilever on mere glass always showed a highly similar adhesion within the measurable range, any deviations due to contamination could be easily detected. As soon as this control curve showed the characteristic shape as the reference curves on mere glass, the cantilever was considered clean again.

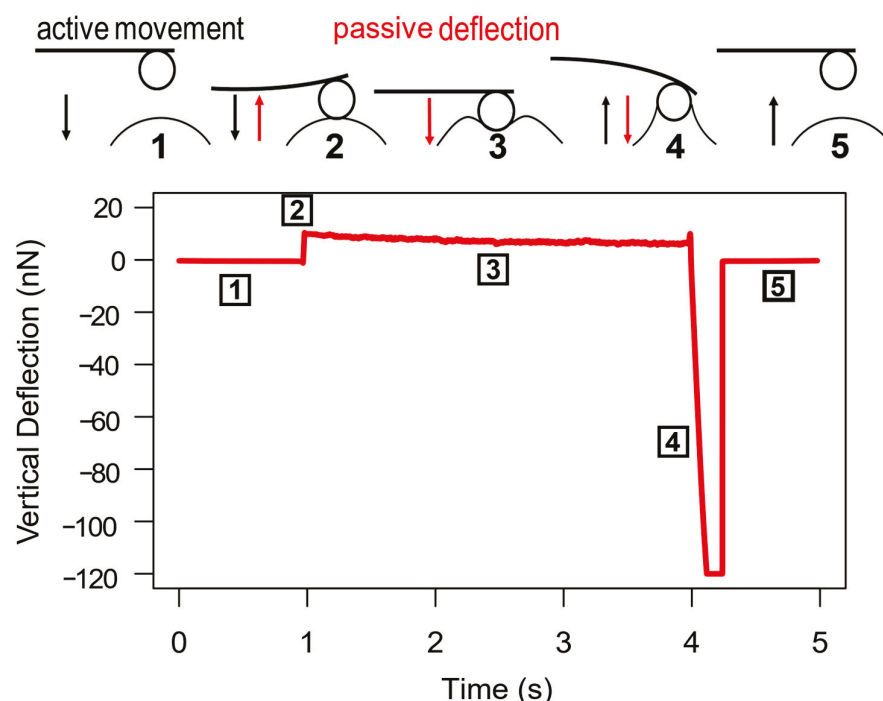


Figure 2. Measurement cycle on “viscous” droplet with reference to the resulting force–time curve. (1) Cantilever approaching droplet. (2) First deflection after contact with droplet’s surface until setpoint force is reached. (3) Delay phase: Stop of cantilever movement and passive deflection caused by droplet deformation. (4) Cantilever retraction and deflection caused by adhesion. (5) Contact breakage and return to the equilibrium position. Black arrows indicate active cantilever movement, red arrows indicate passive deflection without AFM head motion.

2.5. Data Processing

Force curves were plotted using the software JPKSPM Data Processing (version 6.1.172, Bruker Nano GmbH, Berlin, Germany). The bead radius was 5.1 μm . A Poisson’s ratio of 0.5 was used. In all curves recorded on droplets, adhesion was so strong that it exceeded the measurable range and resulted in saturation of the retraction segment of the curve (see Figure 2). A custom script written in MATLAB (version R2025a) (MathWorks) (inspired by Maugis, 1992) [28] was used to fit the initial retraction section of force–distance curves (before saturation) according to the JKR theory. Pull-off force used in the fit was estimated as follows: the saturated section of the curves was extrapolated using two sections (before and after saturation with forces in the range 104–118 nN) by 3rd order polynomial if possible and 2nd order polynomial otherwise. The polynomial minimum was considered to be a pull-off force estimation. Statistical analysis was performed using Sigma Plot 12.0 (Systat Software Inc., San José, CA, USA). The Shapiro–Wilk Test was used to test for normal distribution. Kruskal–Wallis One-Way ANOVA on Ranks and Mann–Whitney Rank-Sum Test were used for comparison of samples. Spearman Rank Order Correlation was used for correlation analysis.

3. Results

3.1. Changes over Time

For investigation of changes in the effective modulus over time, we collected force curves on 13 “viscous” droplets from nine different prints at certain time points (45 min, 60 min, 90 min and 120 min, as well as after 24 h, 48 h and 72 h). JKR analysis provided results for 7 of these 13 droplets, with changes of max. 15% to 25% during up to 48 h only in a few cases, while the majority of measured changes was negligible (Figure S1). All force curves recorded after different periods of time still showed the characteristic properties of the respective droplet category, as previously described. Apparently, the original properties still exceed any further changes within the droplets, even after 72 h. In addition, we analyzed 18 prints by comparing all measurements within the same print, regardless of which droplet was analyzed, but referring only to the time since footprint generation for up to 120 min instead (Figure S2). Following this approach, none of the three measured parameters, namely E-modulus, surface energy or dynamic viscosity, showed any significant correlation with the time since print generation within the first two hours (Spearman Rank Order Correlation test, $\alpha = 0.05$, $p > 0.05$).

3.2. Droplet Dimensions

The maximal droplet height was compared between the two types of pads, arolium and euplantulae, as well as between the treatments of the animals that were used during footprint production (Figure 3C,D). We found comparable heights for both pad types, as well as for all four treatments, with an average over all maximal height of $0.623 \pm 0.561 \mu\text{m}$. Statistical analysis revealed no significant difference between the treatments (Kruskal–Wallis One-Way ANOVA on Ranks, $N_1 = 19$, $N_2 = 16$, $N_3 = 9$, $N_4 = 7$, $DF = 3$, $H = 3.531$, $p = 0.317$). Also, between arolium and euplantulae, there was no significant difference in the height of droplets (Mann–Whitney U test, $N_1 = 38$, $N_2 = 31$, $U = 552.5$, $t = 1048.5$, $p = 0.663$), indicating that the maximal height of secreted droplets is independent from their origin or the used treatment.

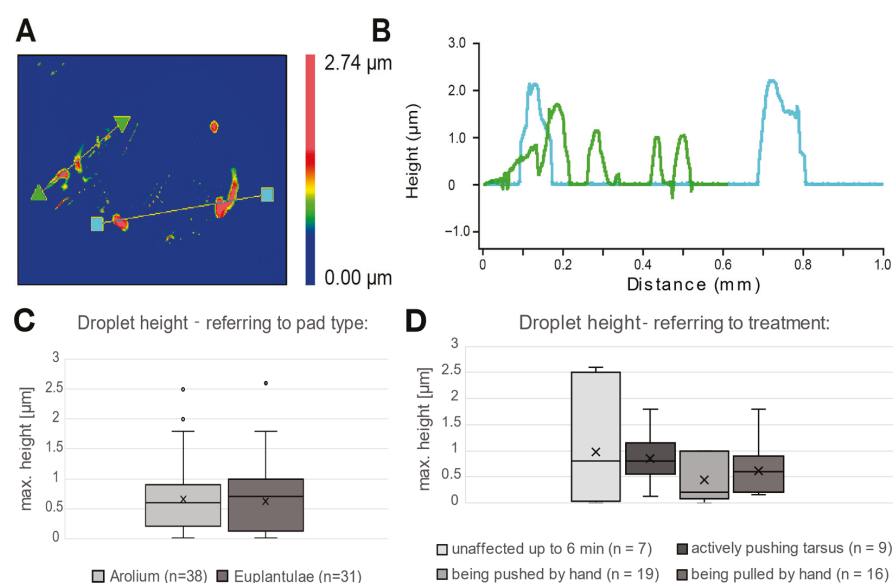


Figure 3. Droplet dimensions. (A) Example of analyzed footprint area, mapping droplet height, with two cross sections (defined by green triangles and blue squares). (B) Height profiles referring to the cross-sections depicted in (A). (C,D) Results of height measurements, with variation in droplets referring to pad type and treatments (n = sample size). Each boxplot displays the median, 25 and 75 percentiles (boxes); 10 and 90 percentiles (whiskers); and the mean is indicated by an “x” symbol.

3.3. Force Measurements of Different Droplets

Based on the force–distance curve shapes and visual observation, the droplets were assigned to three classes: “almost inviscid”, “viscous” and “rigid”. However, the true nature of the “attachment fluid substance” still might be different than these terms suggest. Measurements on “rigid” droplets demonstrated weak jump-in force during contact formation and no distinguishable force decay during delay phase (Figure 4A,B). Visual inspection showed no deformation or other reaction in response to the cantilever indentation. “Almost inviscid” droplets demonstrated strong “jump-in” effect and no distinguishable force decay during delay phase, which means there was no measurable relaxation of the droplet during cantilever indentation (Figure 4C,D). Also, some fluid always stuck to the cantilever in this type of droplet, and so the fluid had to be stripped off after each measurement. “Viscous” droplets demonstrated characteristic relaxation during the delay phase, as shown in Figure 2 (delay phase). During this study, we analyzed 22 “almost-inviscid”, 36 “viscous” and 47 “rigid” droplets. Measurements over time revealed that all three types retained their characteristic properties even over several days. In most prints, only one of these types was observed at once. However, there were a few cases where all 3 types were found simultaneously in the same print.

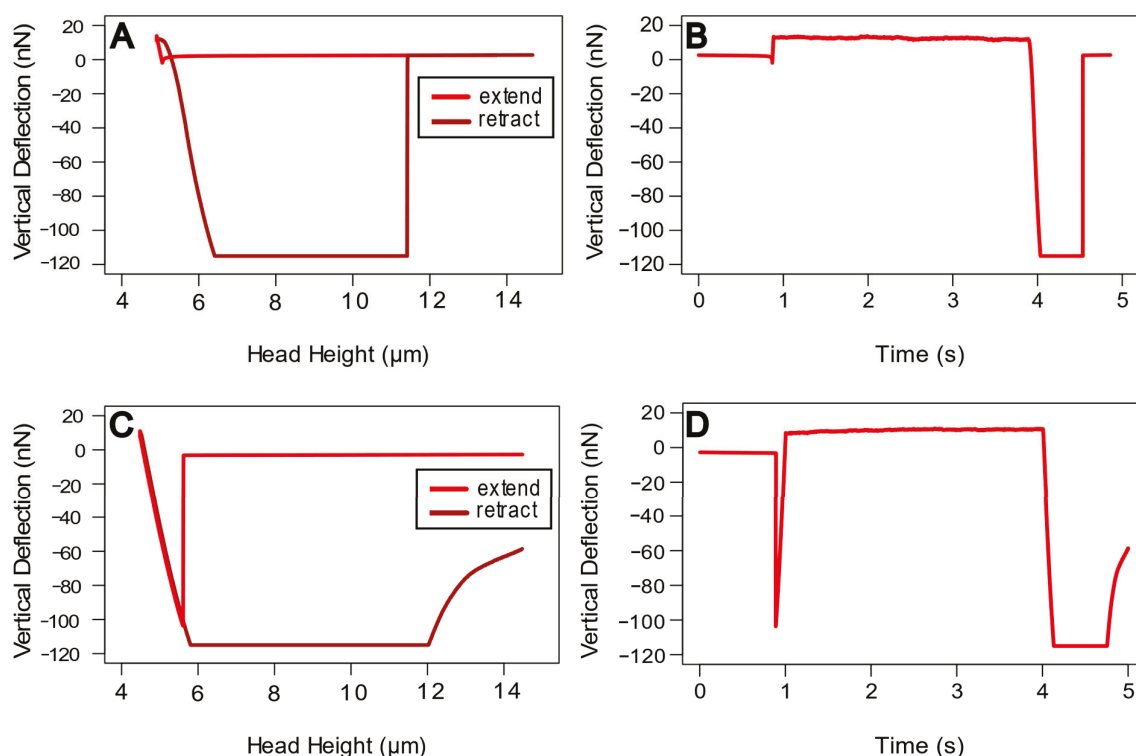


Figure 4. Examples of force–distance (A,C) and force–time (B,D) curves obtained via AFM. (A,B) “Rigid” droplet. (C,D) “Almost-inviscid” droplet, showing strong “jump-in” effect.

3.4. Physical Properties of Droplets

In total, 18 “almost inviscid” droplets, 23 “viscous” droplets and 38 “rigid” droplets were analyzed using the JKR model. For “almost-inviscid” droplets, we measured an average effective modulus of 13.569 ± 2.326 kPa, which was comparable to the result for “viscous” droplets of 13.454 ± 1.932 kPa. For “rigid” droplets, we measured a higher value of 17.258 ± 4.189 kPa. Statistical analysis showed that there was a statistically significant difference between the three groups (Kruskal–Wallis One-Way ANOVA on Ranks, $N_1 = 18$, $N_2 = 23$, $N_3 = 38$, $DF = 2$, $H = 17.382$, $p < 0.001$). Pairwise comparison showed that there was no significant difference between the effective modulus of “almost-inviscid” and

“viscous” droplets (Mann–Whitney U test, $N_1 = 18$, $N_2 = 23$, $U = 184.0$, $t = 401.0$, $p = 0.554$), while both samples were significantly different from “rigid” droplets (Mann–Whitney U test, $N_1 = 18$, $N_2 = 38$, $U = 162.0$, $t = 333.0$, $p = 0.002$; Mann–Whitney U test, $N_1 = 23$, $N_2 = 38$, $U = 194.0$, $t = 470.0$, $p < 0.001$). Furthermore, we calculated the surface energy, and the value for “almost-inviscid” droplets with 11.345 ± 2.410 mN/m and “viscous” droplets with 10.791 ± 0.633 mN/m was higher than that for “rigid” droplets with 9.128 ± 3.352 mN/m. Statistical results for surface energy were similar to those for effective modulus with an existence of significant difference for the three groups (Kruskal–Wallis One-Way ANOVA on Ranks, $N_1 = 18$, $N_2 = 23$, $N_3 = 38$, $DF = 2$, $H = 15.200$, $p < 0.001$), and, respectively, a significant difference between “viscous” and “rigid” droplets (Mann–Whitney U test, $N_1 = 23$, $N_2 = 38$, $U = 213.0$, $t = 937.0$, $p < 0.001$), as well as “almost inviscid” and “rigid” droplets (Mann–Whitney–U test, $N_1 = 18$, $N_2 = 38$, $U = 169.0$, $t = 686.0$, $p = 0.002$), but not between “almost-inviscid” and “viscous” droplets (Mann–Whitney U test, $N_1 = 18$, $N_2 = 23$, $U = 194.0$, $t = 391.0$, $p = 0.743$). The results for effective modulus and surface energy by JKR analysis are shown in Figure 5. In addition, JKR analysis provided a result for the dynamic viscosity of all 23 analyzed “viscous” droplets with a mean value of 17.733 ± 7.226 kPa*s. A summary of all results is provided in Supplementary Material Table S1.

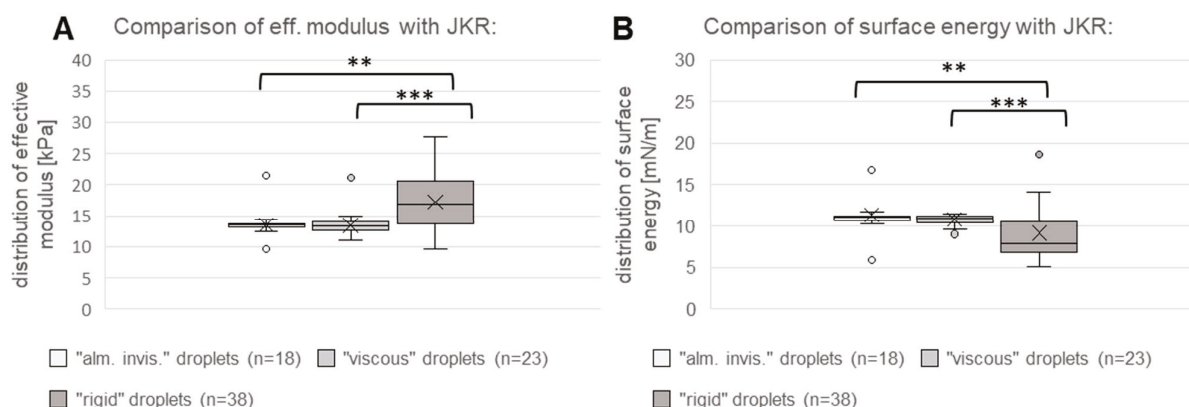


Figure 5. Results of force measurements with JKR model, showing the distribution of effective modulus (A) and surface energy (B) for the three types of droplets: “almost inviscid” (“alm. invis.”), “viscous” and “rigid” (n = sample size). Each boxplot displays the median, 25 and 75 percentiles (boxes); each boxplot displays the 10 and 90 percentiles (whiskers); and the mean is indicated by an “x” symbol. Significance level: ** 0.01–0.001; *** <0.001.

4. Discussion

4.1. Analysis of Viscoelastic Properties

4.1.1. General Challenges of AFM Nanoindentation

The method of nanoindentation on soft biological materials comes with various challenges, as they show different behaviors than the typical materials most commonly examined with indentation methods [29,30]. Furthermore, the convenient models (e.g., Hertz model) require assumptions, including substrate homogeneity, isotropy [31,32] and low adhesion. Especially for biological materials, these assumptions often do not exactly hold due to their complex structure, composition and properties. According to our results, the tarsal fluid examined here is probably more complex and less homogenous than previously assumed [33]. As microscopy analyses of frozen tarsal fluids of another stick insect species (*Medauroidea extradentata*) have shown that the frozen tarsal secretion of stick insects has a different morphological appearance that presumably arises from the different chemical compositions of different fluid droplets [26]. There may be mixture effects of the components inside the fluid, leading to different physical properties, as we found in our results,

depending on the mixture ratios. The surface properties in general can have a strong effect on the contact mechanics compared to the bulk properties of the material [34,35]; therefore, droplets with a high surface-to-volume ratio might behave differently during measurements than those with a low ratio. Furthermore, many previous studies described the thickness of the examined fluid layer and the indentation depth as other critical parameters in nanoindentation experiments. For example, Huth et al. (2019) [32] showed that the calculated effective modulus of the examined probes directly depends on the indentation depth. Another study also investigated the Hertz model for finite thickness of the examined fluid layer, leading to the conclusion that to acquire a good fit accuracy, the indentation depth should not exceed 10% of the total thickness of the fluid layer [36]. In addition, also the total thickness itself can affect the results, as for a very thin fluid layer, the interaction between the cantilever tip and the substrate below the fluid film may become dominant, resulting in an altered viscosity of the fluid between [37].

4.1.2. Evaluation of the Results Using Different Contact Models

The Hertz model does not consider the adhesion between droplet and glass bead, which was even beyond the measurement range of the used cantilever (Figure 4). Thus, the elasticity moduli calculated using Hertz theory would be overestimated, and an analysis with such models as JKR required extrapolation of the force–distance curves to consider the adhesion. The effective elasticity modulus of droplets according to JKR analysis was between 13 and 17 kPa. These values of the attachment fluid elasticity modulus correspond to those for attachment structures provided by previous studies: 250 kPa up to 750 kPa for the whole pad of *Locusta migratoria* (Linnaeus 1758) [38], 27 kPa for the whole pad of *Tettigonia viridissima* (Linnaeus 1758) [8] and 125 to 188 kPa for the arolium of *Medauroidea extradentata*, while for the euplantulae of this species, higher values of 1524 to 1844 kPa were found [39]. Furthermore, the similarities and differences between the three droplet types become more pronounced, as JKR revealed a significant difference between “viscous” and “rigid” droplets. On the other hand, the similarity between “almost-inviscid” and “viscous” droplets was unexpected, considering their different behavior during measurements. It is possible that neither the cantilever nor the used model was sensitive enough to measure the true viscoelastic properties of “almost-inviscid” droplets. Also, the fitting was less precise in the case of “almost-inviscid” droplets compared to “viscous” ones. More detailed analyses in further studies are necessary to determine how strong the effective modulus of solidifying attachment fluid is correlated with its current viscosity. We previously observed that tarsal droplets from some species with smooth pads, like *S. aeta*, *L. migratoria*, etc., could not be analyzed at all with such methods as Brownian motion analysis or Interference Reflection Microscopy (IRM), since the droplets were too viscous and the collection of required amounts of fluid is cumbersome. Consequently, it is reasonable that the nanoindentation method which we used here allowed us to measure the tarsal fluid viscosity in *S. aeta*. The average dynamic viscosity estimated with a generalized Maxwell model [40] was 17.7 kPa*s. Such viscosity value further supports our conclusion that the pad fluid of *S. aeta*, and possibly fluids of smooth pads in general, is more viscous compared to the fluid of previously analyzed species. Previous studies on insect tarsal fluid described far lower values of viscosity between 10 mPa*s and 150 mPa*s for different species of flies, ants and beetles [13,14,41]. However, these studies used different methods, like Interference Reflection Microscopy (IRM) and Brownian motion measurements. The surface energy values according to the JKR model after force–distance curve extrapolation were in the range from 9 to 11 mN/m. This is far below the surface energy of water at room temperature (72.8 mN/m) and fits in the range of various polymers [42]. This rather low surface energy indicates an overall nonpolar nature of the fluid [43], which is in congruence

with the results of previous studies that describe the pad fluid of stick insects as a mainly hydrophobic emulsion with an aqueous dispersed phase [33,41]. The significantly lower surface energy of “rigid” droplets might indicate an even less polar nature compared to “almost-inviscid” and “viscous” ones, which could possibly be explained by the loss of polar volatile components during hardening. It is also generally known that a higher effective modulus of a hard substance goes along with a lower surface energy, which fits well with our results (Figure 5). Furthermore, while we could not measure adhesion directly, the behavior of the cantilever during retraction can be used as a rough indication, considering whether contact breakage with the fluid happened fast, delayed or even not at all after its complete retraction (see Figure 4A,C). Thus, we found significantly higher values for E-modulus and lower values for surface energy, respectively, for curves with a fast contact breakage ($N = 38$) compared to curves with late breakage ($N = 3$) or no breakage at all ($N = 55$) (Kruskal–Wallis One-Way ANOVA on Ranks, $N_1 = 38$, $N_2 = 3$, $N_3 = 55$, $DF = 2$, $H = 22.796/H = 24.400$, $p < 0.001$). This result fits our assumption that E-modulus should decrease and surface energy should increase with adhesion increase. However, direct measurements of adhesion would be necessary to confirm these results. As a conclusion, it can be said that analysis of force–distance curves is challenging, as liquid and hard substances have different requirements for evaluation, making viscoelastic materials, which may contain both components, even more problematic for such measurements. Hui and Baney (1998) [44] investigated the JKR theory in the context of these challenges and concluded that the model can be used for this kind of analysis but comes along with some difficulties in determination of surface energy. Nevertheless, previous studies described similar values of 26 to 27 mN/m for the congruent parameters surface tension and work of adhesion of insect tarsal fluids [9,43], in accordance with our results.

4.1.3. Influence of Droplet Size and Cantilever

Based on our WLI measurements, the average maximal thickness of the secretion was $0.623 \pm 0.561 \mu\text{m}$. For AFM, only droplets of at least $10 \mu\text{m}$ in diameter were used for measurements; in most cases, they exceeded $10 \mu\text{m}$. Therefore, all droplets were characterized as flat-shaped with a high surface-to-volume ratio. However, the strong variations in diameter and height might contribute to the variation in effective modulus and dynamic viscosity. The indentation depth was not identical between droplets due to the method. Nevertheless, the average indentation depth of force curves used for viscosity determination was approx. 60 nm. Considering the assumed average height of $0.6 \mu\text{m}$, this falls well within the 10% limit described by Dimitriadis et al. (2002) [36]. That study also investigated the influence of the shape of the cantilever tip, leading to the conclusion that usage of sharp tips can result in overestimation of the effective modulus, while spherical tips with a radius of $2 \mu\text{m}$ or $5 \mu\text{m}$ delivered values which were comparable to macroscopic measurements. Accordingly, the bead radius that we used should have no significant influence on the results. Referring to the phenomenon of decreasing viscosity described by Darwiche et al. (2013) [37], the interaction between bead and glass slide might also have affected the results, as they found that effect already at distances of several micrometers. However, this is relativized by the fact that they used a much larger bead of $115 \mu\text{m}$ in diameter.

4.2. Characterization of Droplet Types

4.2.1. “Almost-Inviscid” Droplets

Existence of three different droplet types demonstrating distinct characteristic force–distance indentation curves was the most intriguing result of this study. The typical properties of “almost-inviscid” droplets were a remarkable jump-in effect during contact

formation, as well as absence of relaxation during the delay phase. Both features indicate that the cantilever was probably pulled down by a capillary bridge and penetrated the droplet with the bead contacting solid phase or substrate. The phenomenon of capillary bridges between a thin fluid on a flat surface and a cantilever tip has been investigated in several studies during the last decades [45,46] and depends on several factors, including tip shape and radius, as well as contact angles and even the atomic structure of the tip [47]. If some factors, like the wettability of the bead, can be compensated, it might be possible to measure even lower values of viscosity in this category of droplets.

4.2.2. “Viscous” Droplets

On “viscous” droplets, viscoelastic relaxation was obviously observed during the delay phase, and the jump-in effect was much lower in contrast to fluid droplets. Several studies recorded a similar viscoelastic effect on different substrates, using AFM nanoindentation [48–51]. However, none of these substrates has been described as pure fluid. Instead, they have been described as soft materials, hydrogels and polymers, indicating that there has always been a component of macro-molecular structure from simple polymer chains up to complex cellular matrices of living cells. As the “viscous” droplets demonstrated similar rheological properties, it is likely that the tarsal fluid here consists of at least two physicochemically different compounds, as hypothesized by Thomas et al. (2023b) [26] for the tarsal secretion of *Medauroidea extradentata*.

4.2.3. Time Dependence of Elastic Modulus and Viscosity

“Viscous” droplets were the only type from which we observed the effective modulus and viscosity variation after different periods of time after production. Our results revealed that both parameters show inhomogeneous changes, leading to both decrease (in most cases) and increase (in a few cases). Considering the assumed complex semi-fluid composition of this droplet type, it seems that the proportion of elastic and fluid components is changed over time in some cases. It is known that the mechanical properties of biological materials and viscoelastic materials in general depend on basic physical conditions, including time, temperature and even loading rate [52,53]. In addition, the complex composition of insect tarsal fluid [15,18] might facilitate further changes over time. External factors, like temperature and humidity, were kept as constant as possible during the measurements and should not cause any effect. Still, in general, a change in water content can affect the modulus, leading to an increase in the case of evaporation [26] or a measurable decrease in the modulus in response to increasing humidity due to water uptake, as in biopolymers like cellulose [34]. On the other hand, chemical reactions like polymerization or depolymerization can also affect the rheological properties, like the decrease in viscosity due to the enzymatic hydrolysis of soluble cellulose derivatives [54]. However, it is important to note that the measured modulus and viscosity changes over time do not lead to category change by any droplet. Therefore, one can assume that these changes are relatively small and probably do not play an important role for the character of temporal attachment and detachment events in this insect species.

4.2.4. “Rigid” Droplets

The properties of the tarsal fluid become more intriguing, considering the phenomenon of “rigid” droplets. The time between footprint generation on the glass slide and the first measurement on a droplet with the AFM did not exceed three minutes, but still some droplets were already hardened. Whether this solidification process was physically or chemically based could not be deduced from the results. To the best of our knowledge, the chemical composition of the tarsal fluid from *S. aeta* has not been investigated yet. However, previous studies on the stick insect species *Carausius morosus* Brunner 1907

described the tarsal fluid of stick insects in general as an emulsion of volatile hydrophilic microdroplets in an oily continuous phase [33,41]. Emulsions in general can show complex rheological behavior depending on their composition. In this context, evaporation rate plays an important role. Previous studies have already examined some differences in composition and evaporation rate between attachment fluid from beetles and flies, showing a comparably larger fraction of volatile components in the fly fluid [15]. A follow-up study revealed that the fluids of both examined species also differed in their viscosity [14]. In the case of *S. aeta*, viscosity might also depend on the number of hydrophilic components in the fluid; however, we did not observe any detectable evaporation during the experiment in the respective droplets. While some droplets disappeared within 24 h, as could be seen using light microscopy, the “rigid” droplets retained their size even for several days. This observation is in congruence with the findings of Thomas et al. (2023b) [26] for the fluid of *M. extradentata*, which consists of components with different morphologies in the frozen state and includes droplets with different evaporation rates, also including such compounds that show no evaporation at all. In addition, the evaporation observed by Peisker and Gorb (2012) [15] happened over at least 1 h. Considering this time span, it is unlikely that the fast solidification process that we observed was caused by a loss of volatile components. Previous studies examined the chemical composition of tarsal fluid from different species, describing several components, including peptides, proteins and hydrocarbons [18,55]. One conclusion was that components like chain and branched alkenes will result in a semi-solid, grease-like consistency of the fluid [56]. However, to the best of our knowledge, a fast solidification like the one we observed has not been described for any insect species yet.

4.2.5. Variation in Secretion Amount

In general, the occurrence of different droplet types was not consistent over the timeframe of the measurements, but the reasons for this remain unclear. During WLI measurements, we tested different treatments for footprint production, and although only the maximal height was measured, the results indicated that the amount of secreted fluid was independent from the treatment or particular tarsal segment. Furthermore, a previous study on *C. morosus* revealed that the tarsal secretion is independent from body size, indicating that growth or aging of the animals also has no significant effect on the key physical properties of the fluid [25]. During our study, all surrounding conditions were kept constant as far as they could be controlled. In principle, the contact with different plant surfaces can affect the attachment performance in general [57] and might also affect the tarsal secretion if the animals are able to recognize and respond to changes in the surface they encounter. Further investigations may address the question of how widespread the phenomenon of temporal adaptations among insect attachment is and which conditions can cause it.

4.2.6. Possible Ecological Functions of the Fluid Complexity

It is known that the attachment structures of insects are well adapted to various kinds of surfaces. Especially in the euplantulae of stick insects, this adaptation is achieved by specific surface microstructures, which can differ strongly even between closely related species [58]. Previous studies showed that these structures are functionally relevant for the specific ecological preferences of different stick insects, as for example, smooth pads perform stronger on smooth substrates, while nubby pads are adapted to a broader range of surfaces [59,60]. More specific patterns might be an adaptation to wet substrates or specific food plants [61]. However, the role of attachment fluid has not been considered during these investigations. Thomas et al. (2023b) [26] provided results that the tarsal

fluid of *M. extrudentata* can be categorized into four morphologically distinct components, which presumably also differ in their viscosity due to their possible difference in chemical composition. According to these findings, the components with low viscosity produce thin films with large areas, which might contribute to the adaptation to very fine surface roughness. On the other hand, highly viscous thick films may adapt to rather coarse roughness [26].

Previous studies assumed that the complex composition of insect tarsal fluid enables fine tuning of viscosity and other properties as an additional response to various challenges which come along with the substrates in their habitat [16–18,26,62–64]. For example, the two-phasic emulsion of *C. morosus* enhances resistance against shear forces, depending on the amount of hydrophilic microdroplets [33]. The chemical composition of fluid in cockroaches comes with various functions in terms of adhesion, friction or contact-area maximization [65], and the lower viscosity of fluid in flies compared to beetles results in faster contact formation, as well as faster detachment, which might be important in order to escape predators [14]. In addition, the self-cleaning properties of both smooth and hairy pads can also be improved by the fluid, as it can wash off contaminating particles or at least fill the gaps between them to enhance the contact area [16,63]. The natural habitats of stick insects also have a great diversity of surfaces, and therefore they are well adapted to various surfaces and topographies [66]. As part of this adaptation, the properties of the attachment fluid might also be changeable in response to surrounding conditions. In a previous study, *S. aeta* was observed to be rather agile compared to another stick insect species [67]. Also during this study, the animals often showed lots of motion during footprint production, trying to escape. Nevertheless, as the movements of *S. aeta* are far slower than those of beetles or flies, a low-viscosity of tarsal fluid might be beneficial while walking on planar substrates, favoring “almost-inviscid” droplets. On the other hand, climbing on branches or leaves requires more resistance against shear forces, which could be better provided by “viscous” droplets. Finally, it is known that a semi-solid state of fluid can decrease adhesion and allow faster detachment [68]. Whether this might be the function of “rigid” droplets or not needs to be investigated in future studies.

It is known that the two types of attachment pads in stick insects have different functionality for adhesion. The arolium on each pre-tarsus is used to withstand pull-off-forces, and the euplantulae on the other tarsal segments are used to withstand shear forces [69]. Thus, it is reasonable to assume that the properties of attachment fluid differ between both types of pads. In our experiment, we were not able to ascertain the corresponding pad type for every examined print. However, we had 19 droplets that certainly originated from the arolium and 57 droplets that originated from the euplantulae. Almost all of the arolium droplets belonged to the “rigid” type, while the majority of euplantulae droplets belonged to the “viscous” type. Thus, the significant difference in effective modulus and surface energy between both droplet types also results in a significant difference between both types of attachment pads, indicating that the arolium secretes more fast-hardening liquid with high effective modulus and low surface energy. However, these results might not be representative, as we were not able to consider all droplets for this comparison. Further studies might address this question by using a true separation between both pads during footprint acquisition. We also made the observation that “rigid” droplets are easily dissolved in distilled water, indicating hydrophilic properties of at least some components of the fluid, which is in contrast to the generally assumed nonpolar nature of the attachment fluid. One previous study examined humidity as a critical factor on the properties of artificial insect-inspired fibrillar adhesive pads. The authors found that the amount of liquid secreted from the pad had a strong influence on the adhesion at low humidity, while at high humidity, additional effects, like capillary forces caused by humidity and

humidity-induced softening of the pad material, strongly increased the adhesion [70]. In nature, *S. aeta* is found in moist tropical habitats [71,72]. Therefore, in a surrounding with enhanced humidity, the attachment fluid, and especially the “rigid” droplets, might show rather different properties.

5. Conclusions

This first attempt to analyze the mechanical properties of insect attachment fluid from smooth pads with atomic force microscopy revealed a remarkable complexity of the rheological properties of the tarsal fluid of *S. aeta*. Previous optical and macroscopic investigations on the fluid as one system did not differentiate properties caused by single subunits of this system. The qualitative analysis of the fluid indicated that the rheological properties of individual components within the secretion result in three different categories of droplets, “almost inviscid”, “viscous” and “rigid”, which tend to keep their characteristics even for several days. Thus, it is evident that the attachment fluid is a heterogenous mixture. The next step, in addition to verification and improvement of the current results, should be a detailed analysis of the chemical composition of the fluid to search for a correlation between specific components or internal reactions and the rheological properties found here. Furthermore, we were not able to analyze the adhesion properly, as we needed to extrapolate the curves. It would be great to record complete force curves, as analysis of adhesion might reveal phenomena like pull-out of material or step-wise changes. Future studies could address this question by using a stiffer cantilever. A comparison to other stick insect species might provide insights into ecological adaptability of tarsal fluids, and long-term studies in other insect species might reveal adaptive responses of their tarsal fluids to changes in environmental parameters.

One general theorem in the field of biomimetics is that biological systems have the advantage that they are multifunctional, highly adapted and optimized in millions of years of evolution. However, this comes along with a high complexity and a short lifetime compared to technical solutions [73]. Therefore, understanding these complex properties and their behavior is essential to improve their possible practical utilization. The mechanisms relying on specific wet adhesion are already used as part of bio-inspired solutions in lots of different fields [68], including medicine, for example, as part of wound treatment [74] and wearable monitoring devices [75]. Other possible applications lie in the field of bionic robots and in industrial engineering fields, such as marine anti-fouling [76] and anti-icing [77]. The examination of mechanical properties of insect tarsal fluid with AFM nanoindentation can contribute to further insights in this progress, as well as to a better understanding of the remarkable attachment abilities of insects in general.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/biomimetics11010042/s1>, Figures S1 and S2: Changes in effective modulus and surface energy over time. Table S1: Raw data table: height measurements, effective E-modulus and dynamic viscosity.

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Article

Bioinspired Swimming Robots with 3D Biomimetic Shark Denticle Structures for Controlled Marangoni Propulsion

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Abstract

Shark skin exhibits a well-defined multilayered architecture, consisting of three-dimensional denticles and an underlying dermal layer, which contributes to its passive drag reduction. However, the active drag reduction mechanisms of this interface remain largely unexplored. In this study, the Marangoni effect potentially arising from the active secretion of mucus on shark skin is investigated. A 3D-printed swimming robot with a porous substrate and a biomimetic shark denticle structure is developed. By introducing surfactants into the porous substrate and adjusting denticle arrangements, on-demand propulsion and controlled swimming trajectories are achieved. A superhydrophobic surface is fabricated on the swimming robot, which reduces water resistance and enhances propulsion. Moreover, denticles with a 30° attack angle demonstrate optimal propulsion performance in both Marangoni-driven hydrodynamics and aerodynamics. This study suggests that the secretion of mucus on shark skin may facilitate active drag reduction via the Marangoni effect, offering novel insights into the biomimetic structural design of autonomous swimming robots.

Keywords: shark denticles; Marangoni propulsion; superhydrophobic; drag reduction

1. Introduction

Over the past two decades, significant research efforts have been directed toward developing untethered miniature robots, ranging from micrometers to centimeters in size, capable of autonomous movement [1,2], controllable manipulation [3,4], and remote communication [5]. These small-scale robots hold immense promise for applications such as environmental monitoring [6], sensing [7,8], drug delivery [9,10], environmental toxin remediation [11,12], and energy generation [13,14]. Inspired by the intricate locomotion systems of insects, engineers have designed innovative robots that replicate these natural mechanisms. For instance, rove beetles such as *Microvelia* and *Stenus* can glide across water surfaces by secreting chemicals from their rear, creating a surface tension gradient wherein a liquid with lower surface tension is drawn by the surrounding higher surface tension liquid, resulting in rapid Marangoni propulsion [15]. This bioinspired principle has driven the development of self-propelled swimming robots, powered by diverse actuation methods, such as magnetic [16–18], thermal [19–21], electric [22–24], and concentration fields [25–28]. However, existing Marangoni-propelled swimmers consisting of single materials fall short of achieving multiple functionalities, and structural design has emerged as a critical factor

affecting the performance of Marangoni-propelled swimmers, particularly in manipulating surface tension dynamics. While current efforts in structural design have been directed toward the whole swimmer through combining different components, there remains a lack of in-depth investigation into the individual structural elements and their micro-nanoscale features, which are essential for optimizing propulsion and functionality [29].

In nature, organisms secrete a variety of chemical substances to perform diverse functions. Fish, for instance, release mucus on their surfaces to reduce drag, and sharks similarly produce mucus, though current research suggests that the amount secreted is minimal and may not significantly contribute to drag reduction [30–32]. Studies on shark drag reduction have predominantly focused on the passive effects of their denticles [31,33–45], with minimal attention devoted to active mechanisms involving mucus (see Table S1 for a comparison of drag reduction approaches across different studies). However, some evidence indicates that the mucus secreted by fish exhibits a low surface tension, typically ranging from 20 to 30 mN/m [46–49], which is comparable to that of certain chemical fuels, such as ethanol with a surface tension of 22.39 mN/m. This similarity suggests that sharks may utilize an active drag-reduction strategy through the secretion of a specific quantity of mucus from their skin and gills. The low surface tension of this mucus could induce Marangoni propulsion, facilitating active drag reduction. When combined with the hydrodynamic properties of the denticle micro-structures, this mechanism could potentially yield unexpectedly enhanced performance, warranting further investigation into such bioinspired active drag-reduction approaches.

Here, to investigate the potential Marangoni effect induced by mucus on shark skin, we utilized simple and efficient 3D printing technology to fabricate a swimming robot featuring a porous substrate topped by a denticle array structure. By introducing a surfactant through the pores between the denticles, Marangoni propulsion was achieved as the surfactant was released. Through careful adjustment of the denticle arrangement and incorporation of corresponding fluid diversion channels, untethered propulsion and controlled directionality of motion were achieved. Additionally, water resistance was significantly reduced by integrating superhydrophobic denticles and fences along the robot's sides, further enhancing propulsion efficiency. Denticles designed with a 30° attack angle exhibited optimal propulsion performance in both Marangoni-propelled hydrodynamics and aerodynamics. This study introduces a novel concept of biomimetic design for autonomous swimming robots, highlighting the possible role of shark mucus in active drag reduction through the Marangoni effect.

2. Materials and Methods

2.1. Fabrication of 3D-Printed Self-Propelled Swimmer

Freshly deceased specimens of a shortfin mako shark (*Isurus oxyrinchus*) and a Pacific spadenose shark (*Scoliodon macrorhynchus*), were sourced from fishermen in Shandong Province, China. A small piece of skin, measuring $2 \times 3 \text{ mm}^2$, from the flank region of the specimen was used for micro-CT scanning (SKYSCAN 1272, Bruker, Billerica, MA, USA). A single representative denticle was selected from the scanning sample, and a 3D digital mesh model was constructed using Mimics Research 21.0 (Materialise Inc., Leuven, Belgium).

The reconstructed denticle model was duplicated and arranged in a staggered and overlapped pattern and linear pattern, similar to real shark denticles, where the riblet tips of the anterior denticle fall into or interlock with the valleys of the posterior denticle, onto a substrate using 3ds Max (Autodesk, Inc., San Francisco, CA, USA). The real denticle height (1.1 mm) was reduced due to its embedded height in the substrate. The denticles and porous substrate ($3 \text{ cm} \times 3 \text{ cm} \times 2 \text{ mm}$) with diversion walls were printed using a commercial

digital light processing printer (Mini 8K, Phrozen Tech., Taiwan, China) with an X-Y axes resolution of 22 μm and Z-axis resolution of 10 μm , using a methyl methacrylate-SiO₂ mixed resin. The printing layer height was set at 20 μm , with an exposure time of 2.4 s. After 3D printing, the samples were immersed in ethanol for 10 min and subjected to ultrasonic cleaning to remove uncured resin. To enhance the mechanical properties, a post-curing process was performed with LEDs emitting 405 nm light for 3 min at room temperature. Due to limitations in printing resolution, all 3D-printed denticles were scaled up to ten times the actual size of real denticles. To obtain the swimmer with superhydrophobic fences and denticles, the surfaces were carefully brushed with a superhydrophobic solution consisting of 1.0 g of superhydrophobic nanosilica particles (Aerosil R202, average particle size 14 nm, Evonik Degussa Co., Essen, Germany) in 100 mL of n-hexane using a hairbrush and dried under ambient conditions for 10 min to ensure the solvent was completely volatilized.

2.2. Characterization

SEM images were obtained using a field-emission scanning electron microscope at 10 kV (SU8010, Hitachi, Ibaraki Prefecture, Japan). The sharkskin samples were first rinsed in an ultrasonic cleaner with water for 30 s and then cleaned with deionized water three times to remove mucus, dirt, and blood on the surface. Tissue samples were then fixed with 2.5% glutaraldehyde overnight at 4 °C. After fixation, the samples were rinsed with phosphate buffer solution and then rinsed with deionized water three to five times. Dehydration of the tissue was performed by the application of a consecutive series of increased concentrations of ethanol (30%, 50%, 75%, 90%, and 100%) for 15 min each in order to avoid shrinkage and deformation. Finally, the samples were air-dried under a vacuum before being sputter-coated with gold. The water contact angles were measured using a video-based contact angle measuring device (OCA 25, Data-physics, Filderstadt, Germany) with droplets of 2.0 μL . The surface morphology of the 3D-printed Pacific spadenose shark denticles was characterized by OLYMPUS OLS-4500 (Olympus Corporation, Tokyo, Japan). An atomic force microscopy (AFM) image was obtained using an atomic force microscope (Dimension FastscanBio, Bruker, Billerica, MA, USA).

2.3. Propulsion Performance Analysis

For hydrodynamic locomotion, the swimmer was placed in a water tank with an area of 50 $\times$ 50 cm². Locomotion of the swimmer was recorded with a Nikon D750 camera (Nikon, Tokyo, Japan) from the top view. Tracker software (7.6.21) was used to obtain coordinates of the mass center, symmetric axis, and trajectory curvature of the swimmers. For aerodynamic locomotion, the swimmer was connected to a wall-mounted stress-strain sensor (with an accuracy of 1 mN and a measuring range of 0–10 N) to measure the total propulsion force generated during actuation. A pressure-controlled air compressor was connected to a hole on the top of the swimmer to provide a steady airflow input. The propulsion forces were recorded under various airflow pressures to evaluate the aerodynamic thrust performance and efficiency of the swimmer design.

3. Results and Discussion

3.1. Concept and Design Approach

By integrating the shark denticle structure and cutaneous mucus secretion, we propose here a hybrid swimmer design consisting of a porous substrate infused with surfactant for propulsion and a denticle array equipped with fluid diversion channels for directional control. Figure 1a displays a schematic diagram of a shortfin mako shark (*Isurus oxyrinchus*), with its corresponding denticle structure (Figure 1b). A side view reveals the denticles and skin, where the skin comprises a thin epidermis overlaying a thicker dermis, the latter

divided into a superficial stratum laxum and a deeper stratum compactum. Each denticle consists of a crown, neck, and base, with a distinct cavity region between the skin and the crown. The epidermis, composed of only a few cell layers, is situated between the denticles and contains mucus-producing goblet cells, which are less abundant in the shortfin mako compared to other elasmobranch fishes (Figure 1c). Although the shortfin mako sharks are believed to produce less mucus, the role of this mucus in drag reduction remains unclear. Additionally, studies indicate that denticles can tilt to angles exceeding 50° , providing a passive mechanism to manage flow separation and prevent reverse flow (Figure 1d). This dynamic adjustment of denticle angle during swimming likely influences the distribution of mucus on the denticles, as well as the diffusion rate of mucus into the surrounding water, thereby affecting the overall Marangoni propulsion process. In the following sections, we incorporate these biological features into the design of self-propelled swimming robots to investigate their effects on propulsion performance.

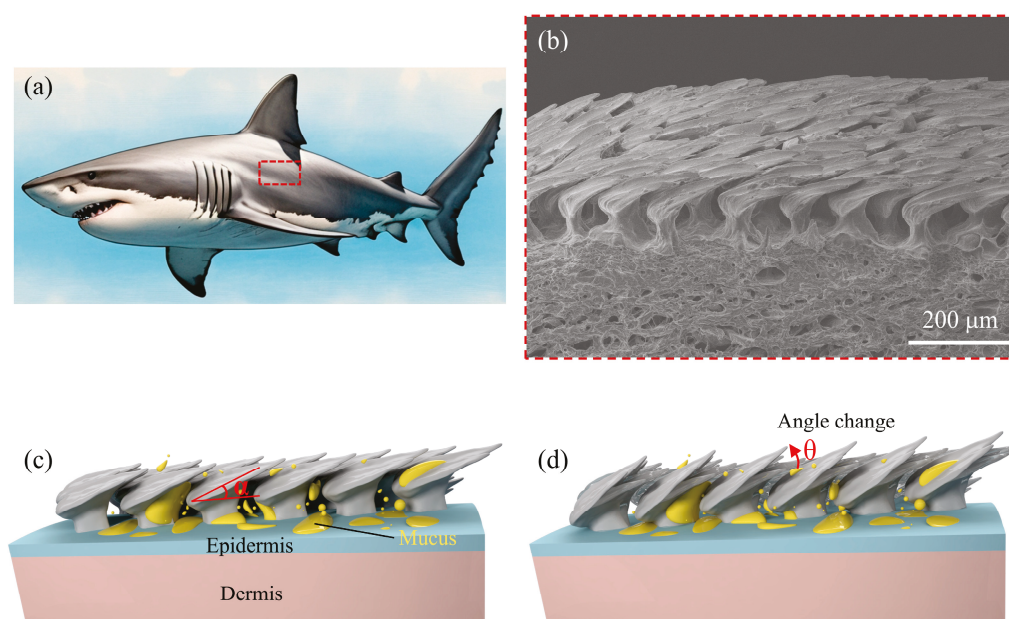


Figure 1. Shark denticle characterization and propulsion design concept. (a) Schematic of the shortfin mako shark (*Isurus paucus*). (b) Cross-sectional scanning electron microscopy (SEM) image of shark denticles. (c) Schematic illustration of the hierarchical structure of shark skin, comprising denticles, epidermis, and dermis, along with the distribution of mucus. Denticles are initially oriented at an attack angle α . (d) Variation in attack angle θ in response to changing flow conditions.

3.2. Design and Hydrodynamics Locomotion of Self-Propelled Swimmers

To fabricate a self-propelled swimmer ($3 \times 3 \text{ cm}^2$) with a denticle array on a porous substrate, we utilized resin-based 3D printing technology to construct a hybrid bilayer structure. The outermost denticle layer was printed using a 3D model reconstructed from micro-computed tomography (micro-CT). Additional details regarding the 3D reconstruction of a single denticle are provided in our previous study [50]. Each denticle features two through-holes beneath it, collectively forming an array aligned with the denticle arrangement (Figures S1 and S2). Figure 2a illustrates the propulsion mechanism of the self-propelled Marangoni swimmer. The 3D-printed swimmer was positioned stationary on the water surface, and a precise volume of surfactant was introduced into the through-holes using a pipette. The surfactant flowed through the holes into the gaps between the denticles, ultimately contacting the water to induce Marangoni convection.

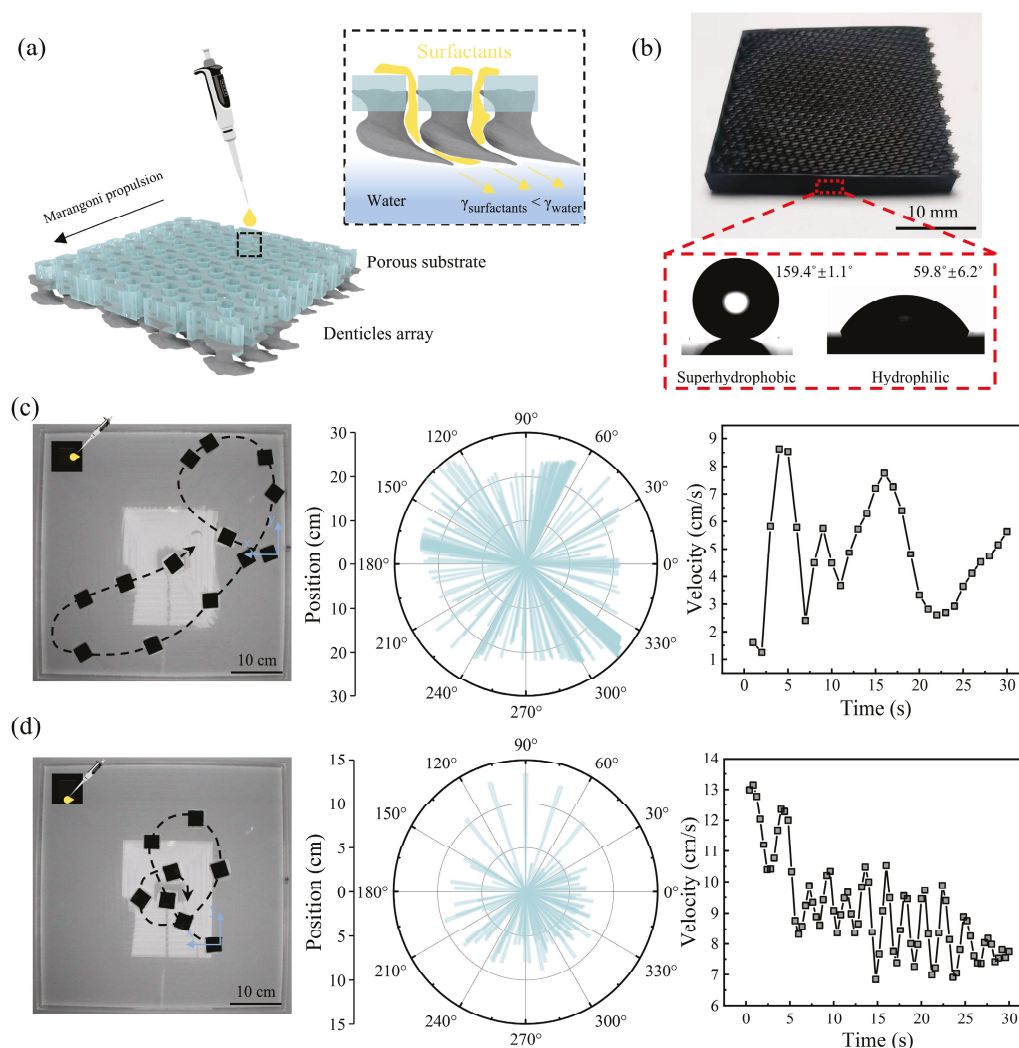


Figure 2. (a) Self-propelled Marangoni-driven swimmer. (b) Photograph of the 3D printed self-propelled Marangoni swimmer with staggered and overlapped denticle arrays, featuring superhydrophobic or hydrophilic fences along sides of the swimmer. (c) Self-propelled Marangoni swimmer with staggered and overlapped denticle arrays exhibits unstable trajectories when HFIP is introduced into the central through-holes on the bottom of the swimmer. (d) Self-propelled Marangoni swimmer with staggered and overlapped denticle arrays exhibits certain clockwise rotational motion when HFIP is introduced into all through-holes in the left half of the swimmer.

To enhance propulsion and control, superhydrophobic fences were incorporated along the sides and head of the swimmer (Figures 2b and S1). The hydrophilic denticles were also rendered superhydrophobic (Figure S3). Initial tests were conducted on staggered and overlapped denticle arrays. When $2.5 \mu\text{L}$ of hexafluoroisopropanol (HFIP) was introduced into the central through-holes on the bottom of the swimmer, the swimmer achieved self-propulsion, but its trajectory was erratic, and its speed fluctuated (Figures 2c and S6a). This behavior resulted from the staggered and overlapped denticle arrangement, combined with the surface tension of the surfactant, causing non-uniform diffusion of the surfactant into the cavity region beneath the denticles. This resulted in heterogeneous surfactant concentrations underneath each denticle, generating non-uniform Marangoni thrust. However, when surfactant was introduced into all through-holes in the left half of the staggered and overlapped denticle array, the swimmer exhibited certain clockwise rotational motion with progressively stabilizing velocity (Figures 2d and S6b). Similarly,

introducing surfactant into the right half of the through-holes enabled counterclockwise rotational motion (Figure S4).

To achieve controlled and predictable motion of the swimmer, we optimized the denticle arrangement. The staggered and overlapped denticle configuration was replaced with a linear arrangement, incorporating diversion walls between every two columns of denticles (Figure S2). This design mitigated non-uniform surfactant diffusion, ensuring that the surfactant remained concentrated within a single column of denticles. The swimmer was divided into 16 denticle columns, and introducing surfactant into the 8th and 9th columns enabled linear forward motion, with the swimmer accelerating initially before maintaining a constant velocity until it reached the edge of the water tank (Figures 3a and S6c). Similarly, adding surfactant to a single column on the left side induced consistent clockwise rotational motion with a progressively decreasing oscillatory velocity (Figures 3b and S6d). Likewise, introducing surfactant to a right-side column resulted in consistent counterclockwise rotational motion (Figure S5).

To enhance the propulsion performance of the linearly arranged denticle swimmer, we rendered the denticles and fences superhydrophobic and compared its performance to that of hydrophilic ones. As the surfactant volume increased, the swimmer's velocity also increased. The superhydrophobic-treated swimmer achieved a higher velocity of 8.63 cm s^{-1} compared to the hydrophilic counterpart (Figure 3c) and sustained propulsion for more than twice as long (Figure S7). We also assessed the propulsion effects of various surfactants, including hexafluoroisopropanol (HFIP), isopropanol (IPA), n-propanol, ethanol (EtOH), and dimethyl sulfoxide (DMSO). As shown in Figure 3d, HFIP, with the lowest surface tension of 14.53 mN/m , yielded the best propulsion performance, while DMSO, with a surface tension of 43.60 mN/m , demonstrated the lowest performance. Table S2 presents the relationship between the surface tension of different surfactants and propulsion velocity, from which an exception is noted with N-propanol and ethanol. Despite ethanol having a lower surface tension (22.39 mN/m) than N-propanol (23.78 mN/m), its propulsion velocity is slower. This anomaly may be attributed to ethanol's faster evaporation rate, which results in a shorter maintenance of the surface tension gradient, leading to rapid dilution of the propulsion region and reduced propulsion force. Additionally, factors such as surfactant wettability, surface morphology, and wetting behavior may also influence propulsion performance. Nevertheless, this indicates that surfactants with lower surface tension result in better propulsion performance. Additionally, we investigated the effect of varying denticle attack angles on propulsion, finding that an angle of 30° resulted in the highest velocity of 10.05 cm s^{-1} , followed by angles of 14° and 45° . Superhydrophobic-treated swimmers consistently outperformed their hydrophilic counterparts across these angles (Figure 3e). Finally, we evaluated both superhydrophobic and hydrophilic swimmers with the five chemical fuels at various attack angles, confirming that the 30° attack angle consistently achieved the highest propulsion velocity regardless of the chemical fuel used (Figure 3f,g). The efficiency of the swimmer is calculated as the kinetic energy of the swimmer (which depends on speed and the total mass of the swimmer) per unit mass of fuel. For the highest-performing swimmer using HFIP as fuel, $2.5 \text{ }\mu\text{L}$ of fuel yielded an efficiency of $1879 \text{ }\mu\text{J g}^{-1}$. The reason for this high efficiency may be that the fuel forms vortices in the cavity region underneath the denticles, enhancing the exchange between fluids. Overall, the fabricated self-propelled swimmer demonstrated on-demand propulsion and controlled swimming trajectories, with optimal propulsion performance at a 30° attack angle. These findings suggest that mucus with a surface tension comparable to chemical fuels, such as ethanol, may generate a Marangoni effect, thereby enabling active drag reduction and propulsion.

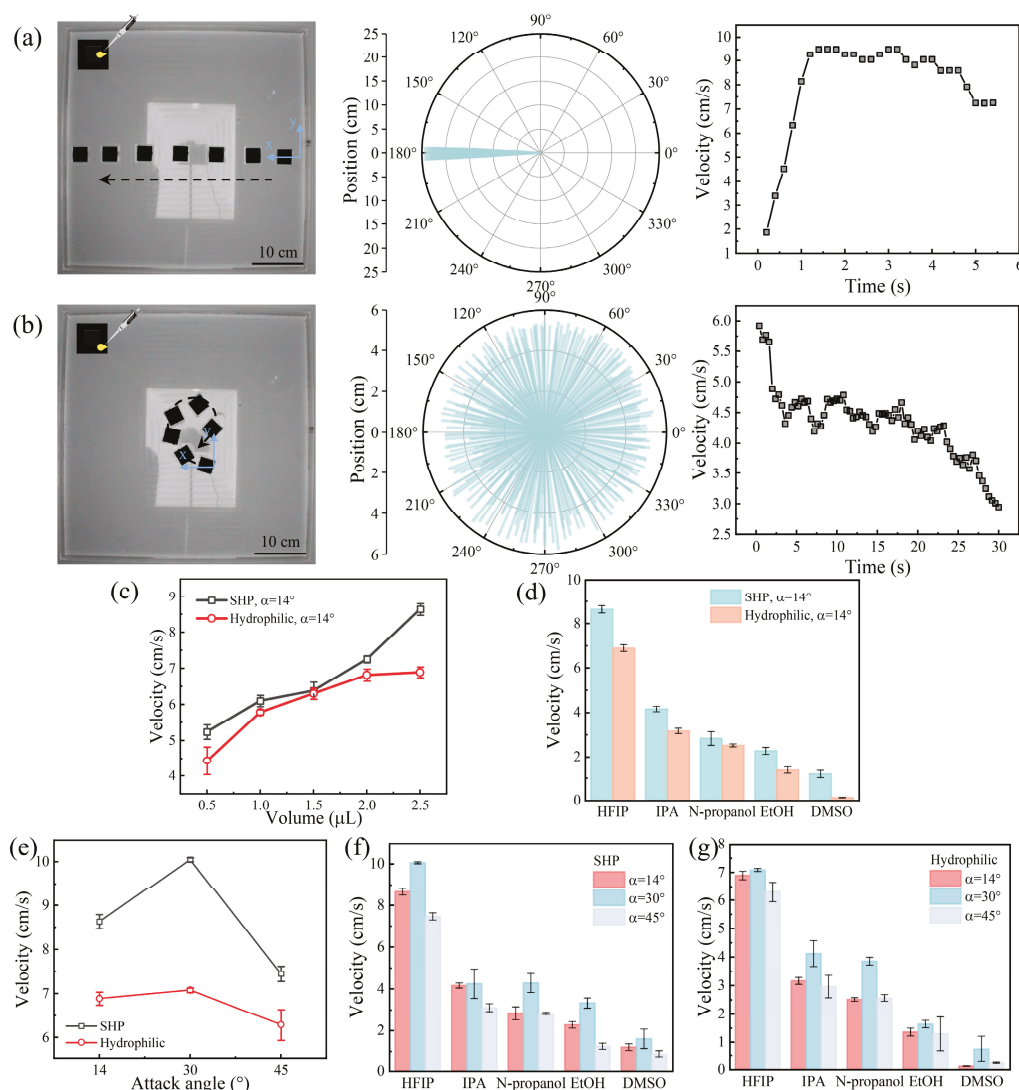


Figure 3. (a) Self-propelled Marangoni swimmer with a linear denticle arrangement exhibiting linear forward motion when HFIP is introduced into the central holes at the bottom of the swimmer. (b) Self-propelled Marangoni swimmer with a linear denticle arrangement exhibiting continuous and regular clockwise rotation when HFIP is introduced into the left column of the swimmer. (c) Propulsion velocity of superhydrophobic and hydrophilic swimmers with a linear denticle arrangement at varying HFIP volumes. (d) Propulsion velocity of superhydrophobic and hydrophilic swimmers with a linear denticle arrangement using various surfactants. (e) Propulsion velocity of superhydrophobic and hydrophilic swimmers with a linear denticle arrangement at varying attack angles. (f) Relationship between the propulsion velocity of the superhydrophobic swimmer and surfactant type at varying attack angles. (g) Relationship between the propulsion velocity of the hydrophilic swimmer and surfactant type at varying attack angles.

3.3. Design and Aerodynamics Locomotion of the Swimmers

To evaluate the propulsion performance of the swimmer under aerodynamic conditions, thrust generation was measured. An air inlet was incorporated at the bottom center of the previously developed Marangoni self-propelled swimmer, which connected to an air compressor. Compressed air entered an internal chamber within the swimmer, flowed through the through-holes beneath the denticles, and was discharged between them. A stress–strain sensor was mounted at the anterior end of the swimmer to measure thrust magnitude (Figure 4a). By varying air pressure, we observed that thrust increased with elevated pressure, with the maximum thrust achieved at a denticle attack angle of

30°, followed by angles of 14° and 45° (Figure 4b). These results, consistent across both hydrodynamic and aerodynamic conditions, confirm that a 30° attack angle yields optimal propulsion performance. And the greater the external propulsion force (air pressure or fuel), the more significant the propulsion effect. Additionally, we investigated the propulsion performance of another shark species, the Pacific spadenose shark (*Scoliodon macrorhynchus*), and found it to be inferior to that of the shortfin mako shark (*Isurus oxyrinchus*) (Figure 4c). This difference may be attributed to the larger denticle size of the Pacific spadenose shark relative to the shortfin mako (Figure S8a,b), resulting in a reduced pore density per unit area

$$\varepsilon = N_{pores} / A \quad (1)$$

where A is the total surface area of the swimmer and N_{pores} is the number of pores. The pore density for the shortfin mako shark was 126 pores cm^{-2} , while that for the Pacific spadenose shark was 79.3 pores cm^{-2} , suggesting that higher pore density facilitates greater interaction between air and denticles, thereby generating greater thrust.

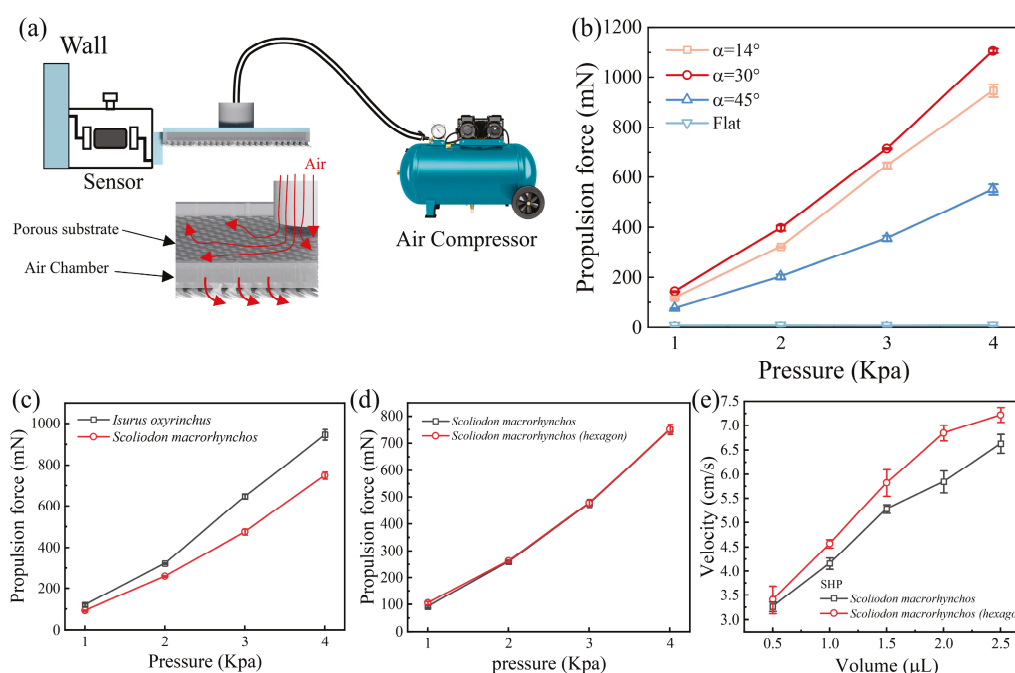


Figure 4. (a) Schematic illustration of the aerodynamic propulsion of the swimmer. (b) Relationship between propulsion force and air pressure at varying attack angles. (c) Relationship between propulsion force and air pressure for denticle models of different shark species. (d) Relationship between propulsion force and air pressure for *Scoliodon macrorhynchus* denticles (with and without hexagonal structures). (e) Relationship between propulsion velocity and HFIP volume for *Scoliodon macrorhynchus* denticles (with and without hexagonal structures).

Finally, further investigation into the propulsion performance of the Pacific spadenose shark revealed a distinct hexagonal pit structure on its denticles, with pit heights ranging from 2–3 μm (Figure S8c,d). The effect of these hexagonal pits on drag reduction or propulsion remains uninvestigated. To investigate this, we fabricated biomimetic denticles with hexagonal structures using 3D printing (Figure S8e,f) and evaluated their propulsion performance. Compared to denticles without hexagonal structures, those with hexagonal pits exhibited no enhancement in propulsion performance under aerodynamic conditions (Figure 4d) but demonstrated improved Marangoni-driven hydrodynamic propulsion (Figure 4e). Subsequently, we tested the drag of denticles with and without hexagonal pit structures in a circulating water tunnel, finding that denticles with hexagonal pits exhibited superior drag reduction (Figure S9). This result may be attributed to reduced

vortex formation [50] in the cavity regions of denticles with hexagonal pits under hydrodynamic conditions. Specifically, when surfactant enters the cavity regions of these denticles, hexagonal pits may retain and direct surfactant flow into the water, facilitating controlled release, suppressing reverse flow, and thereby minimizing vortex formation. In contrast, under aerodynamic conditions, the thrust generated by denticles with and without hexagonal pits was comparable, likely because gases are expelled as jets from the cavity regions under high pressure in both cases. Future studies will focus on the detailed fluidic characterization of surfactant dynamics in the cavity regions and the effects of hexagonal pit structures on Marangoni propulsion and drag reduction. Overall, both hydrodynamic and aerodynamic tests consistently demonstrated that a 30° attack angle provides optimal propulsion performance, with greater external propulsion (fuel or air pressure) enhancing the performance.

4. Conclusions

In summary, we 3D-printed and analyzed the Marangoni propulsion performance of the self-propelled swimmer featuring a porous substrate topped by a denticle array structure, inspired by the denticle structure and mucus secretion of sharks. By introducing surfactants through the pores beneath the denticles, Marangoni propulsion was successfully generated, enabling untethered motion. Optimized adjustments to the denticle arrangement, including the transition from staggered and overlapped to linear configurations, coupled with the incorporation of fluid diversion channels, enabled on-demand propulsion and controllable manipulation over swimming trajectories. The integration of superhydrophobic denticles and fences along the swimmer's sides significantly reduced water resistance, enhancing propulsion efficiency, with superhydrophobic-treated swimmers achieving a maximum velocity of 10.05 cm s⁻¹ and sustaining propulsion for over twice as long as their hydrophilic counterparts. Denticles with a 30° attack angle demonstrated optimal propulsion performance in both Marangoni-driven hydrodynamic and aerodynamic conditions. Comparative analysis of shark species revealed that the shortfin mako shark exhibited superior propulsion performance compared to the Pacific spadenose shark under aerodynamic conditions. Additionally, the hexagonal pit structures on Pacific spadenose shark denticles, with pit heights of 2–3 µm, exhibited notable enhancement in Marangoni-driven hydrodynamic propulsion performance. This study presents a novel biomimetic design for autonomous swimming robots, highlighting the potential role of shark mucus in active drag reduction via the Marangoni effect and providing a foundation for future exploration of micro-nanoscale structural influences on propulsion performance.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biomimetics10080479/s1>, Figure S1: (a) Photography of the top and bottom of the 3D-printed Marangoni swimmer with staggered and overlapped denticle arrays. (b) 3D design rendering of the top and bottom of the Marangoni swimmer with staggered and overlapped denticle arrays, showing superhydrophobic fences along sides of the swimmer and the porous substrate in the bottom; Figure S2: 3D design rendering of the top and bottom of the Marangoni swimmer with a linear denticles arrangement, showing superhydrophobic fences along sides of the swimmer, diversion walls between every two columns of denticles, and the porous substrate in the bottom; Figure S3: Contact angles for hydrophilic and superhydrophobic denticles of the 3D-printed Marangoni swimmer; Figure S4: Self-propelled Marangoni swimmer with staggered and overlapped denticle arrays exhibiting certain counterclockwise rotational motion when HFIP is introduced into all through-holes in the right half of the swimmer; Figure S5: Self-propelled Marangoni swimmer with a linear denticle arrangement exhibiting continuous and regular counterclockwise rotation when HFIP is introduced into the right column of the swimmer; Figure S6: The rotation trace of self-propelled Marangoni swimmer at different times;

Figure S7: Cruise duration of the hydrophilic and superhydrophobic self-propelled Marangoni swimmer (HFIP into the left or right column); Figure S8: Characterization of Pacific spadenose shark (*Scoliodon macrorhynchus*) denticles. (a,b) Side view (a) and top view (b) of 3D characteristics of a representative denticle of a Pacific spadenose shark. (c) SEM image of shark denticles. (d) Atomic force microscopy image of the hexagonal pit structure. (e) Optical image of 3D-printed Pacific spadenose shark denticles. (f) Surface morphology of 3D-printed Pacific spadenose shark denticles; Figure S9: Drag reduction performance of the Pacific spadenose shark denticles (with and without hexagonal structures) in closed circulating water tunnel experiment at a flow rate of 2 m s^{-1} ; Table S1: Comparison of Shark Drag Reduction Methods and Results Across Studies; Table S2: Comparison of Chemical Fuels: Surface Tension and Propulsion Velocity of the Marangoni-Driven Swimmer.

Author Contributions: Conceptualization and methodology, K.Y., R.F., Z.D. and L.J.; software, K.Y. and C.W.; investigation, K.Y. and C.W.; writing—original draft preparation, K.Y.; writing—review and editing, R.F. and Z.D.; visualization, K.Y.; supervision, L.J., R.F. and Z.D.; funding acquisition, L.J., R.F. and Z.D. All authors have read and agreed to the published version of the manuscript.

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Article

Enhancing Legged Robot Locomotion Through Smooth Transitions Using Spiking Central Pattern Generators

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Abstract: In this work, we propose the integration of a mechanism to enable smooth transitions between different locomotion patterns in a hexapod robot. Specifically, we utilize a spiking neural network (SNN) functioning as a Central Pattern Generator (CPG) to generate three distinct locomotion patterns, or gaits: walk, jog, and run. This network produces coordinated spike trains, mimicking those generated in the brain, which are translated into synchronized robot movements via PWM signals. Subsequently, these spike trains are compared using a similarity metric known as SPIKE-synchronization to identify the optimal point for transitioning from one gait to another. This approach aims to achieve three main objectives: first, to maintain the robot's balance during transitions; second, to ensure that gait transitions are almost imperceptible; and third, to improve energy efficiency by reducing abrupt changes in the robot's actuators (servomotors). To validate our proposal, we incorporated FSR sensors on the robot's legs to detect the rigidity of the terrain it navigates. Based on the terrain's rigidity, the robot dynamically transitions between gaits. The system was tested in real time on a physical hexapod robot across four different types of terrain. Although the method was validated exclusively on a hexapod robot, it can be extended to any legged robot.

Keywords: spiking neurons; Central Pattern Generators; legged robots; locomotion; gait transitions

1. Introduction

Legged robots have emerged as a class of robots capable of navigating diverse and challenging environments where wheeled or tracked systems often fall short [1,2]. From uneven terrains to cluttered indoor spaces, legged robots provide superior mobility by mimicking the movement patterns of biological organisms [3]. Their ability to traverse complex surfaces, such as rocky landscapes, stairs, or loose soil, makes them ideal for applications in search and rescue missions, space exploration, and industrial inspection [4]. However, one of the main challenges in developing robust and efficient legged robots lies in achieving smooth transitions between various gait patterns and operational conditions [5,6].

Abrupt shifts in motion, whether due to changes in speed, direction, or terrain type can lead to instability, energy inefficiency, and mechanical stress [7,8].

Legged robots rely on a variety of gaits, which are coordinated patterns of limb movement that allow them to walk, run, jump, or climb [9]. These gaits are typically selected based on environmental conditions and the desired speed or energy consumption of the robot [10]. For instance, walking gaits are preferred at low speeds on rough terrain for stability, while running gaits are more effective for high-speed movement on flat surfaces [11,12]. However, the transition between these gaits, especially in dynamic or unpredictable environments remains a challenge. Poorly timed or abrupt transitions can compromise balance, increase energy consumption, and cause mechanical failures [10]. Achieving smooth, adaptive transitions, such as from walking to running or during surface changes, is thus vital for maintaining the robot's balance, stability, and efficiency [10].

In biological systems, animals transition effortlessly between gaits, adjusting their movement patterns based on terrain and speed [13]. This is enabled by Central Pattern Generators (CPGs), neural circuits capable of producing rhythmic outputs without requiring continuous sensory feedback [14,15]. These circuits are found in the spinal cords of vertebrates and control repetitive locomotion patterns such as walking, swimming, or flying [16]. Inspired by this biological mechanism, researchers have applied CPG-based control systems in robotics to generate smooth and adaptive limb coordination [5]. The use of spiking neurons within CPGs further enhances the biological realism and timing precision of these control systems [17], enabling robots to better emulate the gait modulation observed in animals [18].

Spiking neural networks (SNNs) have demonstrated great potential in bio-inspired robotic control due to their event-based, low-latency communication properties [19–21]. However, most existing studies using SNNs for locomotion rely on large-scale networks or are restricted to simulation environments, requiring high-performance computing platforms for real-time operation [22–24]. These factors limit their use in practical applications, especially on low-cost, resource-constrained robotic systems.

In this paper, we address this gap by introducing a novel, fully bio-inspired spiking CPG controller composed of only 12 neurons capable of producing distinct gait patterns and achieving smooth transitions between them. Gait changes are autonomously triggered based on terrain feedback, using a Force-Sensitive Resistor (FSR) sensor integrated into the hexapod's leg. Crucially, this minimalist architecture enables real-time performance on low-power microcontrollers such as an Arduino board, demonstrating a level of computational and energy efficiency not seen in other SNN-based approaches.

Through simulation and extensive real-world experiments, we demonstrate that our system achieves nearly imperceptible gait transitions, with average stepping time errors below 5 milliseconds, while operating on minimal hardware. These results highlight a path forward for efficient, adaptive locomotion in field-ready legged robots.

The rest of this paper is organized as follows: Section 2 describes the robot architecture, the Spiking Central Pattern Generator (SCPG), the SPIKE-synchronization metric, and the hardware implementation. In Section 3, we present the performance of our approach under various conditions, along with a detailed performance analysis. Section 4 provides a discussion of the results, and finally, Section 5 concludes the paper.

Problem Statement

In [25], a spiking neural network was proposed to reproduce three biologically inspired gait patterns—walk, jog, and run—observed in stick insects, aimed at controlling the locomotion of legged robots. The transition between these gait patterns was managed by initializing a vector that defined the activation of each servomotor in the robot at time zero.

In other words, for each gait pattern, there was an initial vector of pulse values (0 or 1), and from this vector, the spiking neural network, as described by Equations (1) and (2), generated the periodic activation of the neurons (or servos) during the execution of the gait sequence. An example of these vectors is shown in Figure 1. The issue with this approach is that the transition between patterns did not account for the current running time, leading to abrupt shifts that could affect or even damage the servos. This lack of proper synchronization increased the risk of the robot malfunctioning or crashing due to poor coordination between the servos during the transition.

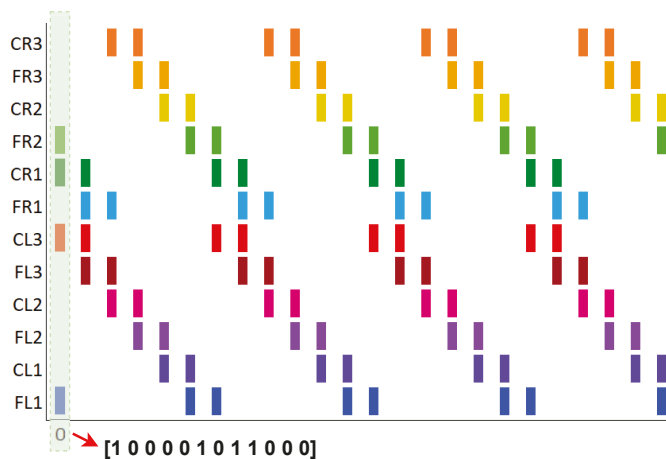


Figure 1. Locomotion pattern “walk”, where each colored line on the graph represents the activation of a servo at a given time. The vertical axis labels the servos (or neurons), with C and F indicating the coxa and femur, respectively, and R and L denoting the right and left sides.

To address this issue, the proposed solution in this research is to enable smooth transitions between gaits by monitoring the activation pulses (spikes) over time and selecting the optimal moment for transitioning. To achieve this, we use an event-based metric called SPIKE-synchronization, which will be explained in detail later.

When the network detects a change in I^{ext} (see Equation (1)) triggered by a sensor input, e.g., an FSR sensor, such as when the robot encounters an obstacle or when the terrain type changes, the system will evaluate the SPIKE-synchronization metric to determine the best timing for the gait transition. This ensures a smoother, more coordinated change, reducing the risk of servo damage or robot malfunction.

2. Materials and Methods

2.1. The Robot

In this work, we consider the hexapod robot shown in Figure 2, which consists of 12 servomotors, 2 per leg, responsible for controlling the coxa and femur. The control system includes an Arduino board and a servo controller capable of sending signals to all 12 servomotors simultaneously. Originally, this robot did not come equipped with any processing system, allowing for the integration of any processing unit. For this project, we selected an Arduino board since the neural network we developed comprises only 12 neurons. Additionally, force sensors (FSRs) have been installed at the base of the robot’s legs to enable it to detect the type of terrain it navigates (see Figure 2). These sensors allow the robot to assess terrain hardness and adjust its gait accordingly.

The following sections detail how the robot’s locomotion gaits are generated and how it interacts with its environment using the FSR sensors.

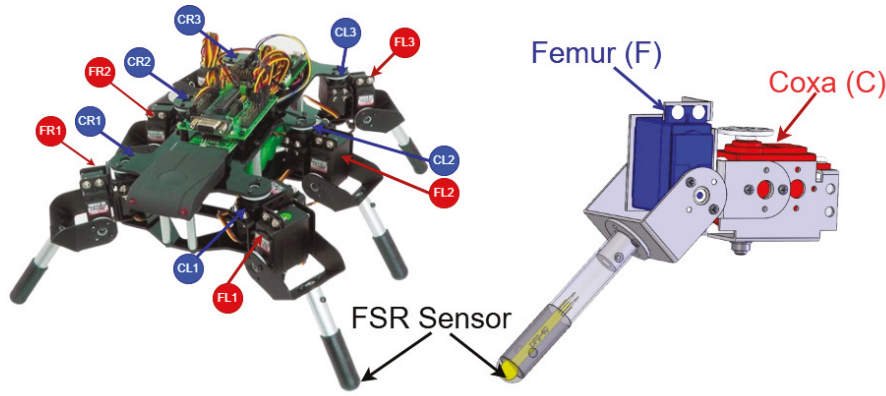


Figure 2. Robot platform overview. The left image shows the hexapod robot structure, with the servomotors controlling the coxa (C) and femur (F) joints highlighted in red and blue, respectively. The labels R and L correspond to the right and left legs. The right image shows a detailed view of a robot limb, showing the femur (F) and coxa (C) servomotors. The coxa joint enables forward and backward movement of the leg, while the femur joint controls the lifting and lowering of the limb. An FSR (Force-Sensitive Resistor) sensor is attached to the distal end of one of the robot’s legs, indicated in yellow.

2.2. Spiking Central Pattern Generators

The robot’s locomotion is achieved through the synchronized activation of its six legs, each powered by two servomotors corresponding to the coxa and femur (see Figure 2), totaling 12 servomotors to control. This control is performed by a bio-inspired mechanism based on a Spiking Neural Network (SNN) (see Figure 3), which acts as a Central Pattern Generator [17], where each neuron corresponds to a servo. The spiking activity generated by the SNN controls the activation and deactivation of the servos, and is modeled by the following equations:

$$V_i[k] = \gamma V_i[k-1](1 - Z[k-1]) + \sum_{j=1}^N W_{ij} Z_j[k-1] + I_i^{ext} \quad (1)$$

and

$$Z_i = \begin{cases} 1 & \text{if } V \geq \theta \\ 0 & \text{otherwise} \end{cases} \quad (2)$$

Equation (1) presents a discretized representation of the electrical activity in the brain, resulting from the interactions and information processing between neurons [26]. This equation is a simplified version of the widely recognized generalized Leaky Integrate-and-Fire (LIF) model [27].

Specifically, Equation (1) describes the behavior of the neuron’s membrane voltage (V). This equation is composed of three key components: the first (in purple) represents the neuron’s internal state, the second (in green) accounts for synaptic activity with other neurons, and the third (in blue) represents an external current (sensory information). In our case, this external current is responsible for triggering the gait transition based on the data collected by force sensors placed on the legs.

In this equation, the value of N is 12, which corresponds to the 6 coxae and 6 femurs of the robot. In this context, this equation describes the membrane voltage generated for the synchronous interaction of these 12 elements. The value of γ is a discharge factor for the neuron, which penalizes the membrane voltage if the neuron has not fired. The firing of the neuron is described by Equation (2), which produces a binary output: 1 when the neuron reaches a threshold and 0 when it does not. In the latter case, the gamma factor comes into play, affecting the voltage. Its value ranges from $[0, 1)$. A value close to 0 indicates a strong impact on the membrane potential, while a value close to 1 results in minimal

penalization. In our case, we considered $\gamma = 0.5$. This value of γ was primarily chosen with digital hardware implementations in mind, as being a power of 2 (i.e., $0.5 = 2^{-1}$) allows for efficient implementation. However, this value can be any number between 0 and 1.

The value of Z in Equation (2) subsequently controls the activation or deactivation of a Pulse Width Modulation (PWM) signal, which drives the robot's movement. This process is explained in Section 2.4.

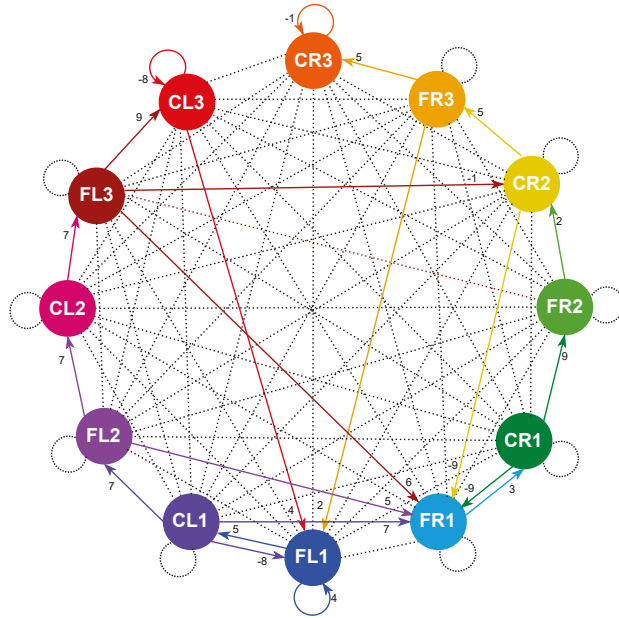


Figure 3. Topology of the Spiking Central Pattern Generator.

$$W = \begin{bmatrix} 0 & 8 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ -7 & 0 & 4 & 2 & -2 & 0 & 1 & 2 & 0 & 0 & 2 \\ -9 & -5 & 0 & 8 & 4 & 1 & 0 & -2 & 4 & 0 & 0 \\ 0 & 0 & 0 & 0 & 8 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 5 & 0 & 0 & 0 & 0 & 0 \\ 6 & 0 & 0 & 0 & -9 & 0 & 0 & 0 & 3 & 0 & -1 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 5 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 5 & 0 & 4 & 0 & -2 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 8 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 9 \\ 0 & -4 & 0 & 0 & 0 & -3 & 0 & -2 & 0 & 0 & 3 \\ 0 & 1 & 0 & -3 & 8 & -1 & 5 & 0 & -4 & 0 & -4 \end{bmatrix} \quad (3)$$

Figure 3 illustrates the schematic topology of the spiking neural network designed for generating three distinct gaits: walk, jog, and run. This topology was developed using a grammar evolutionary algorithm, as detailed in [28]. The synaptic weight matrix employed for creating these three locomotion patterns is given in Equation (3). An important aspect during the estimation of synaptic weights by the algorithm is that they were restricted to integer values only to further reduce the computational cost of implementing the system in hardware, as using only integer weights cuts in half the number of bits required compared to using real values for hardware representation. From this topology, we successfully generated gait patterns, as demonstrated in Figure 4, corresponding to each of the three gaits.

A notable aspect of the patterns observed in Figure 4 is their replication of the gait patterns found in insects, namely tripod and tetrapod gait patterns [29]. According to previous studies, the tripod gait facilitates faster locomotion, as evidenced in Figure 4 (see [30]). In contrast, the tetrapod gait is better suited for medium- and low-speed locomotion.

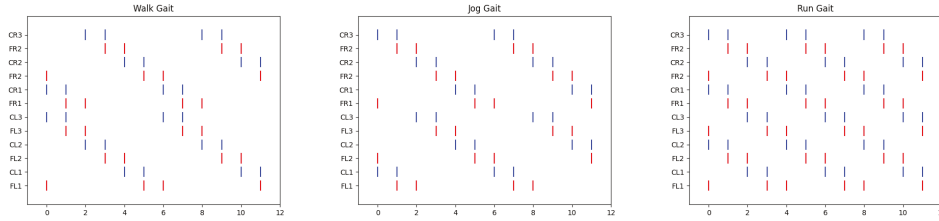


Figure 4. Gait locomotion patterns for different movement speeds: walking (left), jogging (middle), and running (right). Colored lines indicate servo activations (red for the coxae and blue for the femurs).

2.3. SPIKE-Synchronization

Once the different gait patterns are generated, the next step is to evaluate the hexapod robot's ability to transition between them. For this, we use a mechanism known as SPIKE-synchronization, which allows us to assess the current gait state and identify the closest match to the desired target gait. This method applies to transitions aimed at both increasing and decreasing walking speed.

The SPIKE-synchronization is a parameter- and scale-free metric that quantifies the degree of synchrony between events, such as neuronal spikes (see Figure 5a), providing insights into the temporal alignment of these occurrences [31]. This similarity measure produces high values for two similar spike trains; in other words, it reaches a value of one if and only if the two spike trains consist solely of pairs of coincident spikes and a value of zero for spike trains without any coincidences. Let $t_i^{(1)}, i = 1, \dots, M_1$, and $t_j^{(2)}, j = 1, \dots, M_2$, (where M_n is the number of spikes in spike train n , with $n = 1, 2$) be the sequences of discrete spike times. As a starting point, SPIKE-synchronization requires the definition of coincidence detection, which commonly uses a coincidence window $\tau_{ij}^{(1,2)}$ (see Equation (4)), referring to the time lag within which two spikes from $t_i^{(1)}$ and $t_j^{(2)}$ are considered coincident.

$$\tau_{ij}^{(1,2)} = \min\{t_{i+1}^{(1)} - t_i^{(1)}, t_i^{(1)} - t_{i-1}^{(1)}, t_{j+1}^{(2)} - t_j^{(2)}, t_j^{(2)} - t_{j-1}^{(2)}\} / 2 \quad (4)$$

The coincidence specification can be measured using the coincidence indicator in Equation (5), which assigns a value of one to each spike if it is part of a coincidence or zero if it is not; the same rule applies to $C_j^{(2)}$.

$$C_i^1 = \begin{cases} 1 & \text{if } \min_j (|t_i^{(1)} - t_j^{(2)}|) < \tau_{ij}^{(1,2)} \\ 0 & \text{otherwise} \end{cases} \quad (5)$$

Once the coincidence indicator for each individual spike of the two spike trains is defined, the spike trains and their coincidence indicators are aggregated in order to obtain a combined similarity profile by establishing an overall spike index k , producing a consolidated set of coincidence indicators C_k in which each coincidence produces a pair of consecutive ones. The SPIKE-synchronization in Equation (6) is defined as the average value of the profile $C(t_k)$ with $C(t_k) = C(k)$.

$$S_C = \frac{1}{M} \sum_{k=1}^M C(t_k) \quad (6)$$

where $M = M_1 + M_2$ is the total number of spikes in the combined spike trains. S_C can be interpreted as a measure of the fraction of all coincident spikes in the two spike trains; thus,

since the metric is defined as the proportion between the number of spike coincidences and the total number of spikes, it is a dimensionless quantity.

Figure 5b illustrates a transition from jogging to running at time step $k = 3$. At this moment, the SPIKE-synchronization metric reaches the best match for a smoother transition.

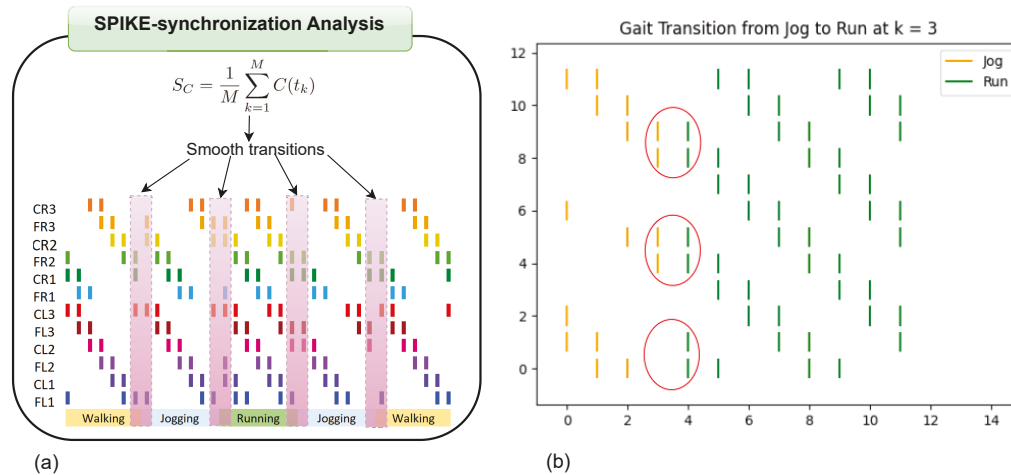


Figure 5. SPIKE- synchronization: (a) general scheme and (b) example of transition from jogging to running at time $k = 3$.

2.4. Hardware Implementation

The Spiking Neural Network used in this study is relatively small, comprising only 12 neurons with a maximum connectivity of 144 when fully connected. However, this is not the case here, as several connection weights are zero (see Equation (3)). For this reason, we opted for a low-cost implementation using an Arduino UNO board. In the future, a neuromorphic system such as SpiNNaker [32] or Loihi [33] could be employed to incorporate a bio-inspired hardware component, as these architectures allow for the direct integration of spiking neuron models.

The 144 possible connections are represented as real (float) values, with each occupying 4 bytes, resulting in a total memory usage of 576 bytes. Considering the Arduino UNO has 32 kB of memory, this implementation utilizes only a small fraction of its capacity.

The other elements in Equation (1) consume minimal resources. The membrane potential requires a real-type variable as well, meaning a total of 12 values need to be stored at each time step, requiring an additional 48 bytes. The parameter γ is a constant value that consumes 4 more bytes. Lastly, Z is a vector of 12 boolean elements.

In total, the memory usage is around 700 bytes, which is approximately 2% of Arduino's total available memory.

On the implementation itself, Equations (1) and (2) have been fully mapped in Arduino, generating a topology as shown in Figure 3, which is in charge of controlling each of the servo motors in the robot (see Algorithm 1). In principle, each of these neurons will be emitting binary pulses, which will represent activation or non-activation of the servo motors in a synchronous manner. However, for this to represent motion in the robot, a PWM signal as shown in Figure 6 must be used.

The conversion of spikes to PWM signals shown in Figure 6 allows the neuronal activity to be translated into control commands for the servomotors. In this case, a direct relationship is established between the firing frequency of the neuron and the duty cycle of the PWM signal. For the femur, when no spike is emitted by the neuron, a PWM signal with a duty cycle of 1500 μ s is generated. However, when a spike occurs, the duty cycle increases to 1900 μ s. In the right coxae, a similar behavior is observed but with inverted

duty cycle values: 1700 μs when the neuron is at rest and 1300 μs during the emission of a spike. On the other hand, in the left coxae, this relationship is inverted, due to the fact that the servomotors are rotated 180° with respect to the right coxae, that is, the duty cycle is lower (1300 μs) when the neuron does not fire and higher (1700 μs) during the emission of a spike.

Algorithm 1 Spiking Neural Network (SNN) update algorithm

```

1: function SNN( $V, Z, W, I^{\text{ext}}, \gamma, \theta, N$ )
2:   for  $i = 1$  to  $N$  do
3:      $\text{sum} = 0$ 
4:     for  $j = 1$  to  $N$  do
5:        $\text{sum} = \text{sum} + W[i, j] \cdot Z[j]$ 
6:     end for
7:      $V[i] = \gamma \cdot V[i] \cdot (1 - Z[i]) + \text{sum} + I^{\text{ext}}[i]$ 
8:     if  $V[i] \geq \theta$  then
9:        $Z[i] = 1$ 
10:    else
11:       $Z[i] = 0$ 
12:    end if
13:  end for
14:  return  $V, Z$ 
15: end function
  
```

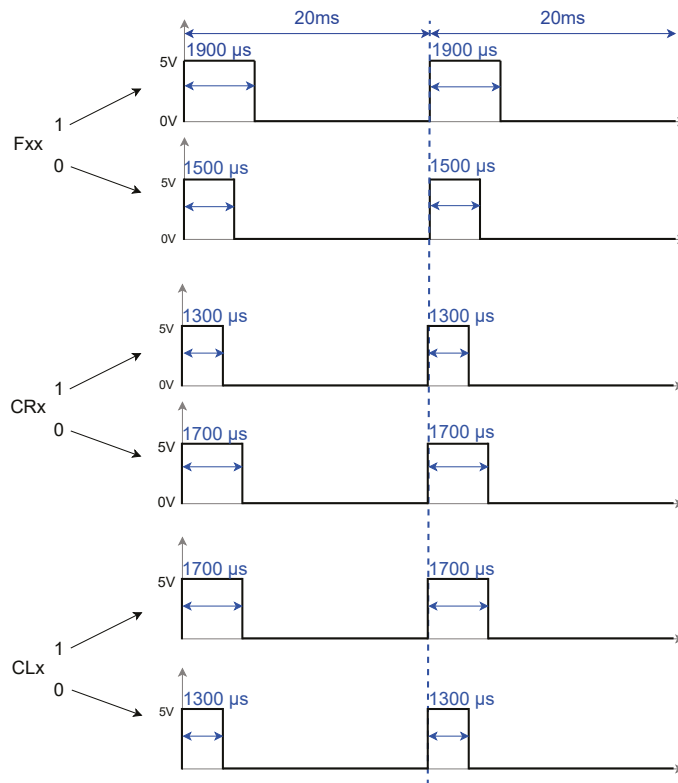


Figure 6. Spikes to PWM.

3. Results

To validate our method, we tested our hexapod robot's navigation across four different surfaces—sand, ground, synthetic grass, and natural grass—as shown in Figure 7. Equipped with Force-Sensitive Resistor (FSR) sensors, the robot is capable of detecting variations in terrain based on changes in resistance measured by the sensors. Table 1

summarizes the mean resistance values observed for each type of terrain. These sensor readings serve as the input for triggering gait transitions in the robot. By identifying specific resistance thresholds associated with each surface, the robot can adapt its gait dynamically.

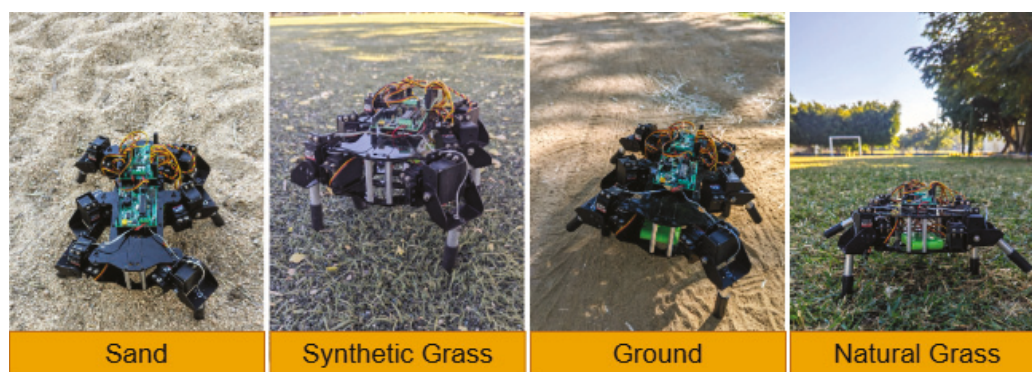


Figure 7. Hexapod robot interaction on regular and irregular surfaces.

Table 1. Mean of 10 FSR readings across the four different surfaces.

	Surface			
	Sand	Ground	Synthetic Grass	Natural Grass
MEAN	14.77 K Ω	6.55 K Ω	9.29 K Ω	33.83 K Ω

SPIKE-synchronization analysis for the different possible gait transitions is presented in Tables 2–6; the rows represent the different patterns at time k for the ongoing gait, and the columns correspond to the possible patterns of the transitioning gait, except for the last column, which indicates the selected time of the transitioning gait that best matches the ongoing gait at time k . Since a SPIKE-synchronization value closer to 1 indicates greater similarity, at each time step k , all potential states of the transitioning gait are evaluated with respect to the ongoing gait in order to identify the one that best matches, thus enabling a smooth transition; in cases where multiple patterns of the transitioning gait reach the highest value, the first one is selected for pairing.

Table 2. SPIKE-synchronization values for times of transition from walk (row) to jog (column) and the specific time to perform the transition. The same distance values are generated for the opposite case (from jog to walk). The values in bold indicate the optimal transition values according to the SPIKE-distance metric.

k	0	1	2	3	4	5	Nearest Time Selected
0	0.00	0.25	0.50	0.50	0.50	0.25	2
1	0.25	0.00	0.25	0.50	0.50	0.50	3
2	0.50	0.25	0.00	0.25	0.50	0.50	0
3	0.50	0.50	0.25	0.00	0.25	0.50	0
4	0.50	0.50	0.50	0.25	0.00	0.25	0
5	0.25	0.50	0.50	0.50	0.25	0.00	1

Table 3. SPIKE-synchronization values for times of transition from walk (row) to run (column) and the specific time to perform the transition. The values in bold indicate the optimal transition values according to the SPIKE-distance metric.

k	0	1	2	3	Nearest Time Selected
0	0.60	0.20	0.20	0.60	0
1	0.40	0.40	0.40	0.40	0
2	0.60	0.60	0.20	0.20	0
3	0.40	0.80	0.40	0.00	1
4	0.00	0.40	0.80	0.40	2
5	0.40	0.00	0.40	0.80	3

Table 4. SPIKE-synchronization values for times of transition from jog (row) to run (column) and the specific time to perform the transition. The values in bold indicate the optimal transition values according to the SPIKE-distance metric.

k	0	1	2	3	Nearest Time Selected
0	0.20	0.60	0.60	0.20	1
1	0.40	0.40	0.40	0.40	0
2	0.20	0.20	0.60	0.60	2
3	0.40	0.00	0.40	0.80	3
4	0.80	0.40	0.00	0.40	0
5	0.40	0.80	0.40	0.00	1

Table 5. SPIKE-synchronization values for times of transition from run (row) to walk (column) and the specific time to perform the transition. The values in bold indicate the optimal transition values according to the SPIKE-distance metric.

k	0	1	2	3	4	5	Nearest Time Selected
0	0.60	0.40	0.60	0.40	0.00	0.40	0
1	0.20	0.40	0.60	0.80	0.40	0.00	3
2	0.20	0.40	0.20	0.40	0.80	0.40	4
3	0.60	0.40	0.20	0.00	0.40	0.80	5

Table 6. SPIKE-synchronization values for times of transition from run (row) to jog (column) and the specific time to perform the transition. The values in bold indicate the optimal transition values according to the SPIKE-distance metric.

k	0	1	2	3	4	5	Nearest Time Selected
0	0.20	0.40	0.20	0.40	0.80	0.40	4
1	0.60	0.40	0.20	0.00	0.40	0.80	5
2	0.60	0.40	0.60	0.40	0.00	0.40	0
3	0.20	0.40	0.60	0.80	0.40	0.00	3

In Figure 8, we illustrate a clear example of the transition from the ongoing gait (jog) to the transitioning gait (run) at early, middle, and late simulation times. Each figure includes a vertical dashed line marking the exact moment of transition from one gait to another.

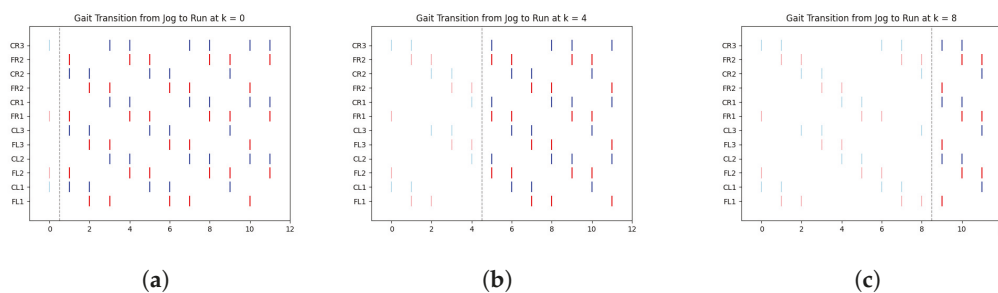


Figure 8. Gait transition from jog to run at (a) early, (b) middle, and (c) late simulation time. These plots present representative examples of neural spike activity during transitions. Red and blue traces correspond to activity in the coxa and femur joints, respectively, reflecting the activation or deactivation of the associated motors.

According to Table 4, Figure 8a shows the jog gait at simulation time 0, which transitions to the run gait pattern at simulation time 1. Similarly, Figure 8b shows the jog gait at simulation time 4, transitioning to the run gait pattern at simulation time 5. Finally, Figure 8c shows the jog gait at simulation time 8, transitioning to the run gait pattern at simulation time 9. The complete set of gait transitions is shown in Appendix A, in Figures A1–A6. A total of 60 experiments were conducted, with 10 trials for each transition type. However, for clarity and better visualization, only five trials per transition are presented.

The dashed line represents the point at which the FSR sensor detected a change in terrain, prompting a gait transition. The spikes are color-coded: red represents the activation of the motor controlling the coxa, while blue corresponds to the femur.

Although the simulation time shown in Appendix A Figures A1–A6 extends up to 12 time steps, these Tables 2–6 display data only up to time step 5. This is due to the periodic nature of the gaits, where the pattern begins to repeat after this point. By focusing on the initial time steps, we can better analyze the effectiveness of the transitions without redundancy.

One particularly interesting observation in these graphs is the shift in balance structure when transitioning between jog/walk and run, or vice versa. During these transitions, the robot switches between tripod and tetrapod balance structures, as described in [29]. This shift highlights the adaptability of the system to changes in terrain and gait demands, demonstrating the robustness of the proposed approach.

Performance Evaluation

To quantitatively evaluate the performance of our approach, Table 7 presents a comparison of the stepping times (in microseconds) during gait transitions in the hexapod robot. The table includes the expected values, the experimentally measured values, and the corresponding errors for each transition. The results show that the average errors range from 3.3 to 4.8 milliseconds, with an overall mean error of 4.043 milliseconds.

Additionally, Table 8 presents the energy consumption, measured in terms of the current, voltage, and power of the electronics and servomotors of the hexapod robot under different gait patterns: running and walking/jogging. The data reflect the energy demands during stable operating conditions at 6 V, and the power was calculated using the equation $P = VI$, where I was measured during execution time.

Table 7. Mean stepping time error between the robot’s legs during each transition under different conditions. The values reflect the temporal synchronization performance of the gait generated by the spiking neural network.

Transition	Stepping Time (μ s)		
	Expected	Measured	Error
Jogging to Walking	1,644,939	1,648,263	3324
Running to Walking	1,644,939	1,649,566	4627
Walking to Jogging	1,244,965	1,248,307	3342
Running to Jogging	1,244,965	1,249,542	4577
Jogging to Running	1,246,622	1,251,403	4781
Walking to Running	1,246,622	1,250,229	3607
mean			4043

Table 8. Energy consumption (current, voltage, and power) of the hexapod robot’s electronics and servomotors under different gait patterns: running and walking/jogging.

Gait	I (mA)	V (V)	P (W)
Running	227	6	13.62
Walking/Jogging	191	6	11.46

4. Discussion

While biological inspiration has long guided research in robotic locomotion, the use of spiking neural networks (SNNs) in this field remains relatively limited. Most studies in gait generation rely on traditional control systems or oscillators, with only a few exploring the unique potential of spike-based, event-driven computation.

Among the existing SNN-based approaches, many employ large and complex networks to generate rhythmic locomotion patterns. For instance, systems such as those implemented on neuromorphic platforms like SpiNNaker or Loihi typically involve hundreds or even thousands of spiking neurons to model Central Pattern Generators (CPGs) or sensory–motor integration modules. Although these implementations have demonstrated biological plausibility and functional control, they come at the cost of high computational complexity and often require specialized hardware.

In contrast, our proposal introduces a fully bio-inspired architecture that operates with only 12 spiking neurons, which is significantly fewer than what has been reported in the literature. This minimalist design is capable of generating and transitioning between three distinct gait patterns—walk, jog, and run—without relying on complex training procedures or extensive synaptic configurations.

A key innovation in our system is the event-based transition mechanism, which is triggered by sensory input from a Force-Sensitive Resistor (FSR). This sensor detects terrain rigidity, allowing the robot to adapt its locomotion speed accordingly. To the best of our knowledge, no previous work has demonstrated such gait transitions driven by event-based sensory mechanisms in SNN-controlled robots.

Furthermore, the low computational demands of our network allow the entire system to run on a low-cost microcontroller platform, such as Arduino, as opposed to power-hungry processors or external compute units. This not only reduces cost and system complexity but also contributes to greater energy efficiency—an essential feature for autonomous, battery-powered robots.

As a future research direction, we envision extending this bio-inspired architecture by incorporating neuromorphic sensors, such as event-based vision sensors (e.g., Dynamic Vision Sensors (DVSs)) or tactile neuromorphic interfaces. These sensors would provide

spiking input natively, enabling more seamless and biologically plausible integration with the SNN controller while also potentially allowing the robot to adapt to more complex and dynamic environments with enhanced sensory resolution and lower latency.

5. Conclusions

The aim of our work is to reduce the gap between basic research, as exemplified in our previous studies, where we successfully designed a unique Spiking Central Pattern Generator capable of generating multiple gaits and applied research, which is the current focus of our efforts to extend the capabilities of our proposal. In this regard, we explored the integration of a transition mechanism between gaits in the locomotion of a hexapod robot, aiming to make these transitions as smooth as possible. To achieve this, we utilized an event-based metric that measures the degree of similarity between two spike trains (neural activations). This metric was incorporated because the robot's locomotion is controlled by a spiking neural network (SNN), which generates three gait patterns (walk, jog, and run) based on events, or spikes. These patterns are produced by a single network capable of transitioning from one gait to another, with the transitions triggered by the FSR readings based on the terrain conditions.

A critical aspect of our approach, as observed in real-world experiments showcasing the robot's gait transitions, is the deliberate modulation of locomotion speed in response to the terrain. Specifically, the robot adjusts its velocity, slowing down or speeding up, based on the rigidity of the surface, as measured by an integrated Force-Sensitive Resistor (FSR) sensor. This adaptive behavior is fundamental to the system, allowing the robot to maintain stability and efficiency when navigating different environments. The observed delay during gait transitions, which reflects the time needed for the robot to adjust its speed, is quantitatively captured and discussed through the stepping time error presented in Table 7. Notably, the transition error is nearly imperceptible, with a maximum average stepping time error of 0.004781 s in the worst-case scenario.

We conducted numerous experiments on a real hexapod robotic platform with a low-cost processing unit, namely Arduino. While not every trial resulted in a perfectly smooth transition, the majority achieved transitions that were nearly imperceptible, highlighting the potential of the proposed approach to improve robotic locomotion performance.

Although experimentation was carried out on a six-legged platform, the methodology is extensible to robots with varying numbers of limbs. Furthermore, integrating neuromorphic platforms such as SpiNNaker or Loihi could enhance the system, advancing toward a fully bio-inspired robotic locomotion framework. This paves the way for more energy-efficient and adaptable robots capable of navigating complex environments with improved robustness and flexibility.

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Data Availability Statement: No new data were generated for this research; however, real-world tests conducted on the robotic platform can be found at the following link: <https://github.com/BioInspiredLab-UGTO/Smooth-CPG> (accessed on 3 June 2025).

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

Appendix A. Gait Transitions

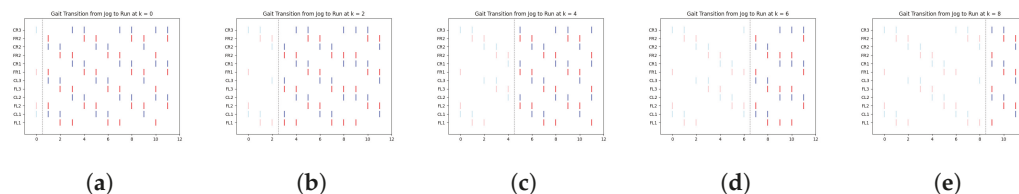


Figure A1. Gait transition from jog to run at different time steps. These plots present representative examples of neural spike activity during transitions. Red and blue traces correspond to activity in the coxa and femur joints, respectively, reflecting the activation or deactivation of the associated motors.

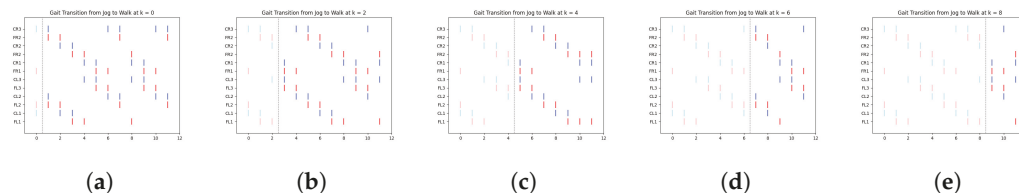


Figure A2. Gait transition from jog to walk at different time steps. These plots present representative examples of neural spike activity during transitions. Red and blue traces correspond to activity in the coxa and femur joints, respectively, reflecting the activation or deactivation of the associated motors.

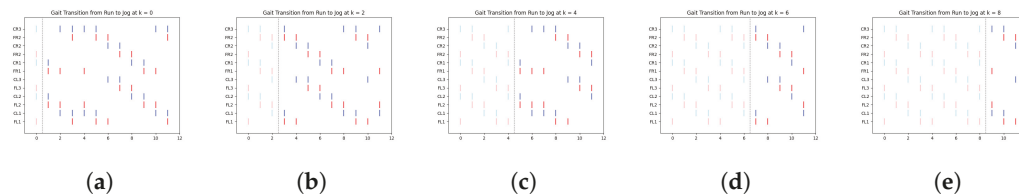


Figure A3. Gait transition from run to jog at different time steps. These plots present representative examples of neural spike activity during transitions. Red and blue traces correspond to activity in the coxa and femur joints, respectively, reflecting the activation or deactivation of the associated motors.

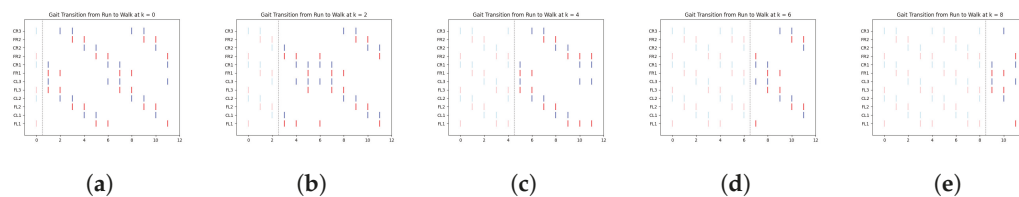


Figure A4. Gait transition from run to walk at different time steps. These plots present representative examples of neural spike activity during transitions. Red and blue traces correspond to activity in the coxa and femur joints, respectively, reflecting the activation or deactivation of the associated motors.

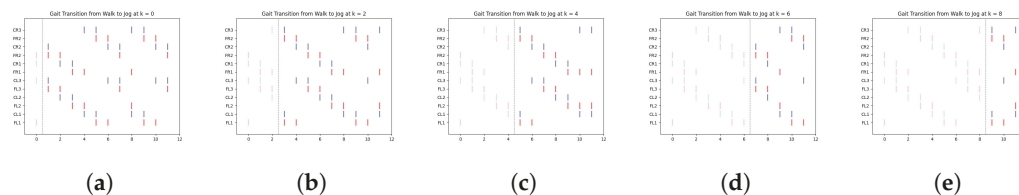


Figure A5. Gait transition from walk to jog at different time steps. These plots present representative examples of neural spike activity during transitions. Red and blue traces correspond to activity in the coxa and femur joints, respectively, reflecting the activation or deactivation of the associated motors.

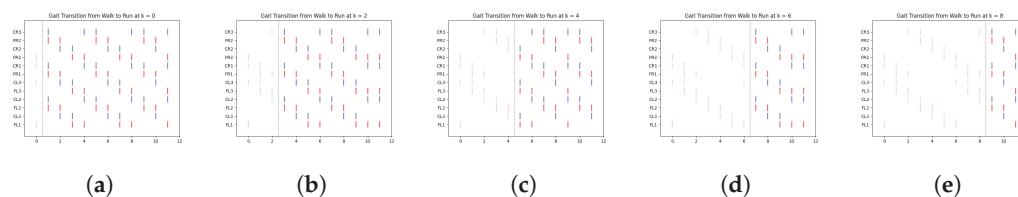


Figure A6. Gait transition from walk to run at different time steps. These plots present representative examples of neural spike activity during transitions. Red and blue traces correspond to activity in the coxa and femur joints, respectively, reflecting the activation or deactivation of the associated motors.

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Article

The *Drosophila* Connectome as a Computational Reservoir for Time-Series Prediction

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Abstract: In this work, we explore the possibility of using the topology and weight distribution of the connectome of a *Drosophila*, or fruit fly, as a reservoir for multivariate chaotic time-series prediction. Based on the information taken from the recently released full connectome, we create the connectivity matrix of an Echo State Network. Then, we use only the most connected neurons and implement two possible selection criteria, either preserving or breaking the relative proportion of different neuron classes which are also included in the documented connectome, to obtain a computationally convenient reservoir. We then investigate the performance of such architectures and compare them to state-of-the-art reservoirs. The results show that the connectome-based architecture is significantly more resilient to overfitting compared to the standard implementation, particularly in cases already prone to overfitting. To further isolate the role of topology and synaptic weights, hybrid reservoirs with the connectome topology but random synaptic weights and the connectome weights but random topologies are included in the study, demonstrating that both factors play a role in the increased overfitting resilience. Finally, we perform an experiment where the entire connectome is used as a reservoir. Despite the much higher number of trained parameters, the reservoir remains resilient to overfitting and has a lower normalized error, under 2%, at lower regularisation, compared to all other reservoirs trained with higher regularisation.

Keywords: reservoir computing; biomimetics; *Drosophila*; neural networks; bio-inspired computing

1. Introduction

In the ever-expanding field of machine learning (ML), increasing performance generally involves increasing model complexity, which in turn lengthens learning time [1–3]. This is typically achieved in one of two ways: either by adding more tunable parameters in neural networks (NNs), such as Transformers, CNNs, or LSTMs [4–7], or by increasing the number of simulations in reinforcement learning and genetic algorithms [8–10].

Other NN architectures, such as those used in echo state networks (ESNs) [11] and liquid state machines (LSMs) [12], form a category of models referred to as reservoir computing, where most of the computation is performed by a randomly connected network of untrained synapses. Reservoir computing is a neural network paradigm that requires minimal training compared to most advanced ML models [13,14]. In a similar fashion to radial basis functions [15], reservoir computing relies on a nonlinear expansion of the dimensionality of the input data, which can then allow the problem to be solved linearly by

a simple fully connected layer known as the readout. A notable difference between the two approaches is the recurrent nature of reservoirs, which adds a temporal buffering behavior to the system [16,17]. This characteristic represents a fundamental advantage in the task of time-series prediction, in which reservoir computing performs exceptionally well [18,19], even in the case of chaotic time-series [20,21].

While the original concept of a reservoir consisted of virtually simulated pools of neurons that act as nonlinear expansions and temporal buffers for time-series, physical implementations spanning several scientific fields have already been implemented, with examples including physical reservoir computing [22,23], photonic reservoir computing [19,24], and even memristor reservoir computing [25,26]. Broadly speaking, depending on the neuronal model used as the activation function [27,28], reservoir computing can be subdivided into liquid state machines, using spiking neurons, echo state networks, based on classical firing-rate neuron models, and time-delay reservoirs, introducing a temporal delay to the neuron dynamics.

The standard practice when creating a reservoir involves randomly initializing a desired number of neurons and synapses, adhering to a specified sparsity, spectral radius, and distribution of non-zero weights [29,30]. While reservoir size, spectral radius, and sparsity significantly influence performance, other parameters often receive limited attention [21,31].

Despite these standardized practices, we still lack an understanding of what constitutes a good reservoir. One promising line of research focuses on exploring physical systems with reservoir-like properties by using various combinations of materials, structures, and processes [32]. For instance, SnO_x -based physical reservoirs have been shown to exhibit key properties observed in biological systems, such as long-term potentiation (LTP) and depression (LTD) [33]. In addition, polymer electrolyte-gated MoS_2 transistors [34] and stochastic memristive devices in the form of a 3D filament matrix [35] have demonstrated reservoir-like behavior suitable for tasks relying on memory and the ability to learn temporal dynamics, where they maintain low error over relatively long prediction horizons for tasks such as chaotic time-series prediction and high accuracy on tasks such as spoken digit recognition.

Nature, on the other hand, has evolved neuronal systems over millions of years in the form of animal brains with a vast diversity of capacities, functions, and specializations [36,37]. The example given by nature is often taken as an inspiration in the field, which in recent years has seen several attempts at bio-inspired and biologically plausible reservoir computing [38], from the imitation of neuronal plasticity [39] to the artificial implementation of the role of both neurons [40] and smaller biomolecules [41].

One study has taken a more drastic approach involving moving away from bio-inspiration and towards biomimicry by mapping the connectivity of connectomes of different species to a reservoir, in an attempt to investigate the performance of the former [42]. However, that study was limited both by the limited range of investigated hyperparameters and by the size of the investigated connectome, as at the time there was no reported case of a complete scan. In particular, whereas the reported result of connectome-based topology and random weights seems to imply a performance at best comparable to the standard fully randomized reservoir, the attempt at also simulating the synaptic weights was unsuccessful, further lowering performance for the heterogeneous connectivity case and completely failing for the homogeneous one. Following that study, connectome topologies have been proposed as an additional constraint on top of standard practice [43].

Similarly, both the topology and the distribution of synaptic weights of several partial connectomes, including humans, have been shown to play a fundamental role in affecting the memory capacity of connectome-based reservoirs. On one hand, the presence of

small-world organization in biological connectomes is shown to increase the resulting memory capacity due to extreme robustness and effective communication [44]. This is achieved with a few hyperconnected neurons that create redundant clusters paired with long-range neurons that quickly transfer information between different clusters. Similarly, Suarez et al. [45] have shown how connectome-based reservoirs perform better near the edge of chaos, thanks both to their topological structure and the distribution of synaptic weights. Whereas these works successfully show that a biomimetic approach to generating reservoirs can increase their memory capacity, they have been performed based on partial connectomes of limited sizes, and they do not directly show how specific reservoir features affect the predicted trajectories when used as time-series predictors.

Nevertheless, thanks to the *FlyWire* project, the complete connectome of a fruit fly brain has been recently published [46]. This represents an important milestone in neuroscience since it is the first complete scan of a relatively large brain. The only other documented complete scan of a connectome is of the *Caenorhabditis elegans*, with only 302 neurons [47], which has been investigated as an AI model [48] but contains too few neurons to be used for a thorough investigation within the field of reservoir computing. This recent scientific advancement provides a unique opportunity to analyze the topology and structure, as well as the role of synaptic weights, of an animal's neural connectivity up to the scale of the entire connectome.

In this work, we explore the possibility of using the topology and synaptic connectivity of the scanned *Drosophila* connectome as a computational reservoir for time-series prediction, comparing its performance to state-of-the-art implementations. Whereas the topology of limited parts of the *Drosophila* connectome, namely the olfactory system [49], has already yielded good preliminary results, the implementation of different sparsities between the biomimetic architecture and the control case, in addition to the limited size of the study itself, prevented a deeper understanding of the relationship between the connectome's features and the observed increase in prediction performance. To the authors' knowledge, this is the first reported study that investigates and characterizes the combination of topology and synaptic weights for an entire connectome and showcases its performance as a time-series predictor. Compared to previous works on connectome-based reservoirs for time-series prediction, we propose a much wider study, both regarding the reservoir's size, including several different subsets of increasing size up to the entire connectome, and the explored hyperparameter space. We also investigate hybrid architectures, which are only partially based on the biological data, in order to characterize the individual effects of connectome topology and synaptic weights, respectively. To achieve this, we perform time-series predictions with an increasing forecast horizon, utilizing multiple different trajectories of a notoriously chaotic nonlinear time-series: the three-body problem [50]. Not only does this problem represent a historical milestone in the investigation of Lagrangian determinism, but it is also characterized by a greater challenge than other chaotic time-series often used for benchmarking since the network is required to predict three time-series simultaneously (the x , y , and z positions of the body). Moreover, it follows recent interest in generative techniques for the n -body problem [51]. Our results demonstrate that the performance of the fly connectome is noticeably different from that of classical ESNs. Notably, reservoirs based on the fly connectome exhibit reduced overfitting in scenarios where overfitting plays a significant role [50].

2. Materials and Methods

2.1. Connectome Extraction

In order to represent the fly connectome as a reservoir, the data of both neurons and synapses were extracted using the CAVE client Python interface provided by the library

caveclient 6.4.1. Neurons and neuronal classes were extracted simultaneously, saving the unique ID, the x , y , and z spatial positions, and the cell class of every single neuron. For reference, Table 1 outlines all the different classes and their labels and briefly describes their function. With the extracted information, it was possible to visualize the *Drosophila* connectome and to group its neurons according to their properties, as shown in Figure 1.

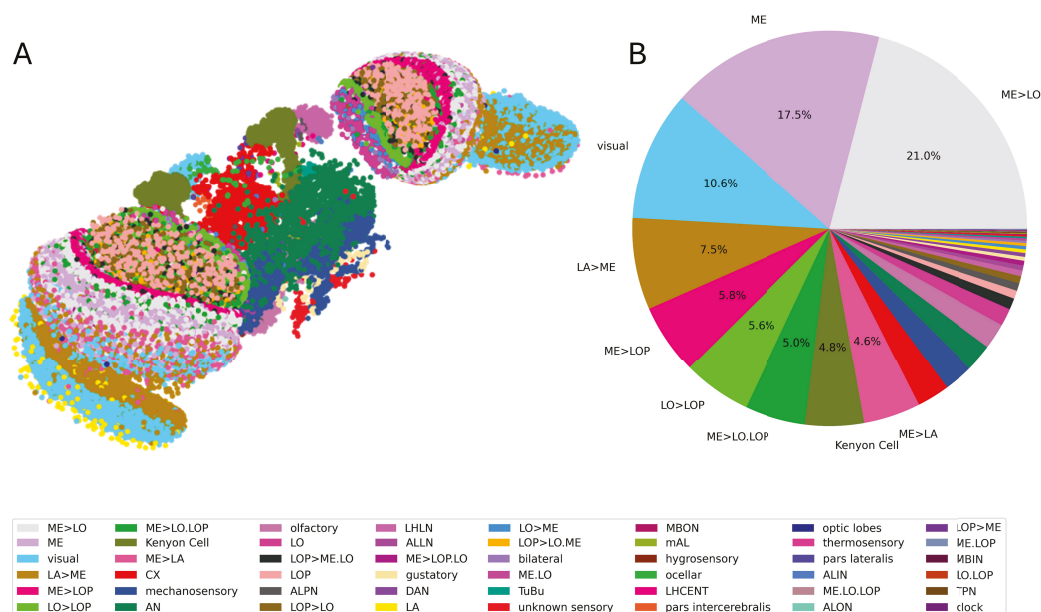


Figure 1. (A) A 3D scatterplot of the entire connectome and (B) a pie chart of the class division within the connectome itself.

In order to create a reservoir, we retrieved all the synapses that had a given neuron as a pre-synaptic neuron for every neuron previously collected. Queries of the database returned the following information:

- The unique IDs of the pre-synaptic and post-synaptic neurons;
- The Cleft score: a measure of how well-defined the synaptic cleft is;
- The Connection score: a measure of the size of the synapse;
- The probability of the synaptic neurotransmitter being Gamma-Aminobutyric Acid, Acetylcholine, Glutamate, Octopamine, Serotonin, or Dopamine.

The first pre-processing of the data was performed by applying a synapse quality filter: a simple threshold-based filtering of the synapses. First, synapses in which the post-synaptic neuron was not within the original set of classified neurons were deleted. Then, all synapses with either a Connection score or a Cleft score below a given threshold were also deleted to avoid false positives. Following the guidelines of the original work [46], we selected these thresholds to be 50 for the Cleft score and 100 for the Connection score. Whereas there are few synapses with a Connection score as high as 10^4 , most of them are between 10^2 and 10^3 .

In order to transform the biological data into a connectivity matrix suitable for a reservoir, we had to determine the weights of the connections between neurons. Unfortunately, the provided database did not provide physiological information, but only anatomical information. Since synaptic strength can only be inferred from direct measurements, in this work, we used the Connection score as an estimate of the strength of each synapse, assuming that larger synapses lead to stronger interactions, as shown for limited neuronal pools in the literature [52,53].

Table 1. Neuronal class abbreviation, complete name, and brief function description for all classes present in the connectome.

Neuron Class	Full Name	Function	Neuron Class	Full Name	Function
CX	Central Complex	Motor control, navigation	Kenyon Cell	Kenyon Cell	Learning and memory
ALPN	Antennal Lobe Projection Neuron	Olfactory signal relay	LO	Lamina Output Neuron	Early visual processing
bilateral	Bilateral Neuron	Cross-hemisphere connections	ME	Medulla Neuron	Visual processing
ME>LOP	Medulla to Lobula Plate	Motion detection	ME>LO	Medulla to Lobula	Higher-order vision
LO>LOP	Lobula to Lobula Plate	Object motion detection	ME>LA	Medulla to Lamina	Visual contrast enhancement
LA>ME	Lamina to Medulla	Photoreceptor signal relay	ME>LO.LOP	Medulla to Lobula and Lobula Plate	Motion and feature integration
ALLN	Allatotropinergic Neuron	Hormonal regulation	olfactory	Olfactory Neuron	Detects airborne stimuli
DAN	Dopaminergic Neuron	Learning, reward, motivation	MBON	Mushroom Body Output Neuron	Readout of learned behaviors
ME>LOP.LO	Medulla to Lobula Plate and Lobula	Visual integration	LOP>ME.LO	Lobula Plate to Medulla and Lobula	Motion-sensitive feedback
LOP	Lobula Plate Neuron	Optic flow detection	LOP>LO.ME	Lobula Plate to Lobula and Medulla	Visual-motor integration
LOP>LO	Lobula Plate to Lobula	Motion-sensitive projection	LA	Lamina Neuron	First visual synaptic layer
AN	Antennal Neuron	Mechanosensory and olfactory processing	visual	Visual Neuron	General vision processing
TuBu	Tubercle Bulb Neuron	Connects brain to vision	LHCENT	Lateral Horn Centroid	Odor valence processing
ALIN	Antennal Lobe Interneuron	Olfactory modulation	mAL	Mushroom Body-associated Antennal Lobe Neuron	Olfactory-learning link
LHLN	Lateral Horn Local Neuron	Innate odor-driven behavior	ME.LO	Medulla and Lobula Neuron	General vision processing
mechanosensory	Mechanosensory Neuron	Touch, vibration detection	ME.LO.LOP	Medulla, Lobula, and Lobula Plate Neuron	Multi-layer vision processing
LO>ME	Lobula to Medulla	Visual feedback	hygrosensory	Hygrosensory Neuron	Detects humidity
pars lateralis	Pars Lateralis Neuron	Hormonal/circadian regulation	unknown sensory	Unknown Sensory Neuron	Uncharacterized sensory function
LO.LOP	Lobula and Lobula Plate Neuron	Motion and space awareness	ocellar	Ocellar Neuron	Light intensity detection
optic lobes	Optic Lobe Neuron	General vision processing	pars intercerebralis	Pars Intercerebralis Neuron	Hormonal regulation
ME.LOP	Medulla and Lobula Plate Neuron	Motion and edge detection	gustatory	Gustatory Neuron	Taste processing
ALON	Antennal Lobe Olfactory Neuron	Olfactory cue processing	MBIN	Mushroom Body Input Neuron	Learning circuit modulation
thermosensory	Thermosensory Neuron	Temperature sensing	clock	Clock Neuron	Circadian rhythm control
LOP>ME	Lobula Plate to Medulla	Motion-sensitive feedback	TPN	Transmedullary Projection Neuron	Optic lobes to brain

Furthermore, information about the synaptic neurotransmitter was used in order to determine the signs of the weights in the connectivity matrix. Among all neurotransmitters, Gamma-aminobutyric acid is considered an inhibitory neurotransmitter, corresponding to a negative weight, and Acetylcholine and Glutamate are considered excitatory, corresponding

to positive ones. Conversely, Octopamine, Serotonin, and Dopamine do not directly act as excitatory or inhibitory, but they instead modulate the resting potential of the post-synaptic neuron. Since ESNs feature a firing-rate neuronal model rather than a spiking one, they do not explicitly represent the resting potential, and thus the synapses that utilize modulatory neurotransmitters were not taken into consideration. For each synapse, we determined the neurotransmitter by taking the one with the highest probability in the database.

The result of this extraction process is a graph with 104,909 nodes and the associated connectivity matrix. Computationally, a reservoir of such a size is too expensive to run: for comparison, most studies focus on ESNs with 10^2 to 10^3 neurons. To obtain a smaller reservoir, we opted to retain a given number, N , of connected neurons, where N is arbitrary. To do this, we implemented two possible selection criteria: ‘most connected’ and ‘proportional’. In the ‘most connected’ case, the N most connected neurons were selected. However, it is important for the fairness of comparison with the control classical reservoir that the selected subset of neurons consists of a single connected graph in order to form a single reservoir of size N rather than two or more independent smaller ones. Therefore, only the largest connected component was considered, resulting in a reservoir of size $M \leq N$. Then, the $N - M$ nearest neighbors were added to obtain the desired size, prioritizing the most connected neurons among all nearest neighbors.

In contrast, the ‘proportional’ criterion attempts to preserve the overall class distribution of the connectome, selecting the N most connected neurons while maintaining the same proportion of each class of neurons with respect to the entire connectome. Then, to obtain a single connected reservoir, the minimum number of nodes needed to connect all selected components were added. The resulting reservoir was of size $M \geq N$. Finally, the least connected $M - N$ nodes were pruned from the graph, obtaining the desired reservoir size. Note that due to the requirement for a single connected subgraph, additional extraction steps were applied after the application of the selection criteria. These steps affected the final selected reservoir, which might differ significantly from the original N initially selected neurons.

Once a reservoir has been defined, its connectivity matrix can be extracted and used for the practical implementation of the ESN. This matrix would have a specific spectral radius, which corresponds to the largest eigenvalue of the connectivity matrix. The matrix is then rescaled with respect to its original spectral radius and multiplied by 0.99. This procedure ensures that the Echo State Property (ESP) is respected and that the reservoir operates near the edge of chaos, as indicated in previous work on time-series [49], which has been shown to improve the performance of both ESNs and biomimetic ESNs [44,45].

To characterize the topology of each extracted reservoir, three standard metrics were taken into consideration: neuron degree, clustering coefficient, and shortest path length. The degree k_i of a neuron i is the number of synapses incident to it, and the degree distribution captures the frequency of each k_i across all neurons. The local clustering coefficient C_i of a node i is defined as follows:

$$C_i = \frac{2e_i}{k_i(k_i - 1)} \quad (1)$$

where e_i is the number of edges between the k_i neighbors of neuron i . The shortest path length ℓ_{ij} is the minimum number of synapses traversed to reach neuron j from neuron i ; we computed the distribution of all ℓ_{ij} with $i \neq j$ across the entire reservoir. To account for directionality, the clustering and degree distributions were computed separately for incoming and outgoing synapses in order to differentiate between pre-synaptic and post-synaptic neurons, and the shortest path was computed considering directionality.

2.2. Echo-State Network

The aim of this work is to investigate the *Drosophila* connectome as a reservoir within an ESN framework. Let us consider the equations governing an ESN (Figure 2):

$$\mathbf{x}_n = (1 - \alpha)\mathbf{x}_{n-1} + \alpha \tanh(\mathbf{W}\mathbf{x}_{n-1} + \mathbf{W}^{\text{in}}[1; \mathbf{u}_n]), \quad n = 1, \dots, T \quad (2)$$

where T is the number of timesteps in the time-series, $\mathbf{x}_n$ represents the reservoir state at timestep n , $\mathbf{W}$ is the reservoir weight matrix, $\mathbf{W}^{\text{in}}$ is the input weight matrix, $\mathbf{u}_n$ is the input signal, and α is a scalar between 0 and 1, so that $1 - \alpha$ is the leakage rate.

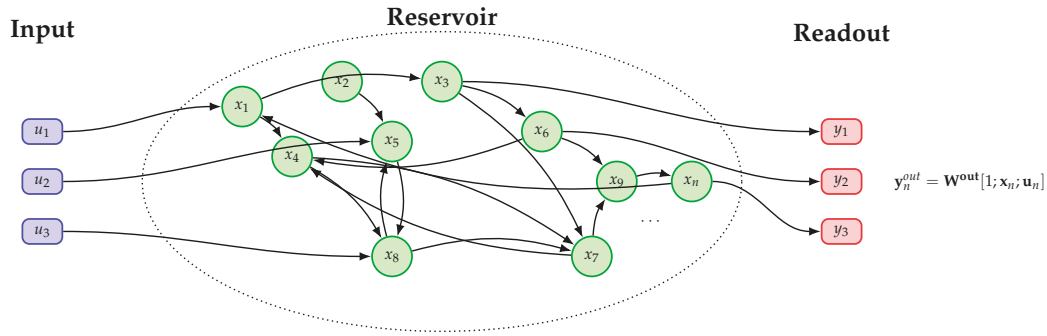


Figure 2. Diagram of an ESN in which a subset of neurons in the reservoir (green) receive the input signal from the input neurons (blue). The readout layer (red), which is usually a fully connected linear layer, extracts activations from another subset of neurons and produces the output.

The output readout layer is defined as follows:

$$\mathbf{y}_n^{\text{out}} = \mathbf{W}^{\text{out}}[1; \mathbf{x}_n; \mathbf{u}_n], \quad (3)$$

where $\mathbf{W}^{\text{out}}$ is the output weight matrix. The objective is to learn $\mathbf{W}^{\text{out}}$ such that $\mathbf{y}_n^{\text{out}}$ closely approximates a given target behavior (signal or label) $\mathbf{y}_n$. By organizing all target readouts into a matrix $\mathbf{Y} \in \mathbb{R}^{N_y \times T}$ and all reservoir dynamics into a matrix $\mathbf{X} \in \mathbb{R}^{(1+N_x+N_u) \times T}$, we obtain

$$\mathbf{Y} = \mathbf{W}^{\text{out}}\mathbf{X}, \quad (4)$$

which constitutes an overdetermined system in the unknown $\mathbf{W}^{\text{out}}$. The least squares solution, which minimizes the mean squared error (MSE), is given by

$$\mathbf{W}^{\text{out}} = \mathbf{Y}\mathbf{X}^T(\mathbf{X}\mathbf{X}^T)^{-1} \quad (5)$$

A regularized solution, obtained via ridge regression [54], which minimizes the MSE along with a penalty on the weight magnitude, is given by

$$\mathbf{W}^{\text{out}} = \mathbf{Y}\mathbf{X}^T(\mathbf{X}\mathbf{X}^T + \beta\mathbf{I})^{-1} \quad (6)$$

where β is the regularization parameter and $\mathbf{I}$ is the identity matrix.

2.3. Time-Series Generation

ESNs are often used for the classification and regression of time-series. In particular, they have been shown to have good performance even when trained on chaotic time-series [21]. In this study, ESNs are tested on the prediction of the circularly restricted three-body problem (CR3BP). CR3BP trajectories were obtained through propagating a set of initial conditions $[\mathbf{r}; \mathbf{v}]$, where $\mathbf{r}$ and $\mathbf{v}$ are the initial x, y , and z positions and velocities, respectively. These trajectories were then generated through propagation using the *heyoka* 6.1.2 library [55], considering the mass ratio parameter of the earth–moon system

and a timestep of 0.1 in the normalized units of time. Each resulting trajectory consisted of 10,000 datapoints and was subdivided into three subsets: washout, training, and test (Figure 3). The washout subset was used as an input to the reservoir with the sole purpose of nullifying the effect of the randomized initial state of the neurons. Then, the training set was fed to the reservoir to collect the data necessary for the aforementioned one-shot training, and the test set was used to evaluate the performance of the ESN.

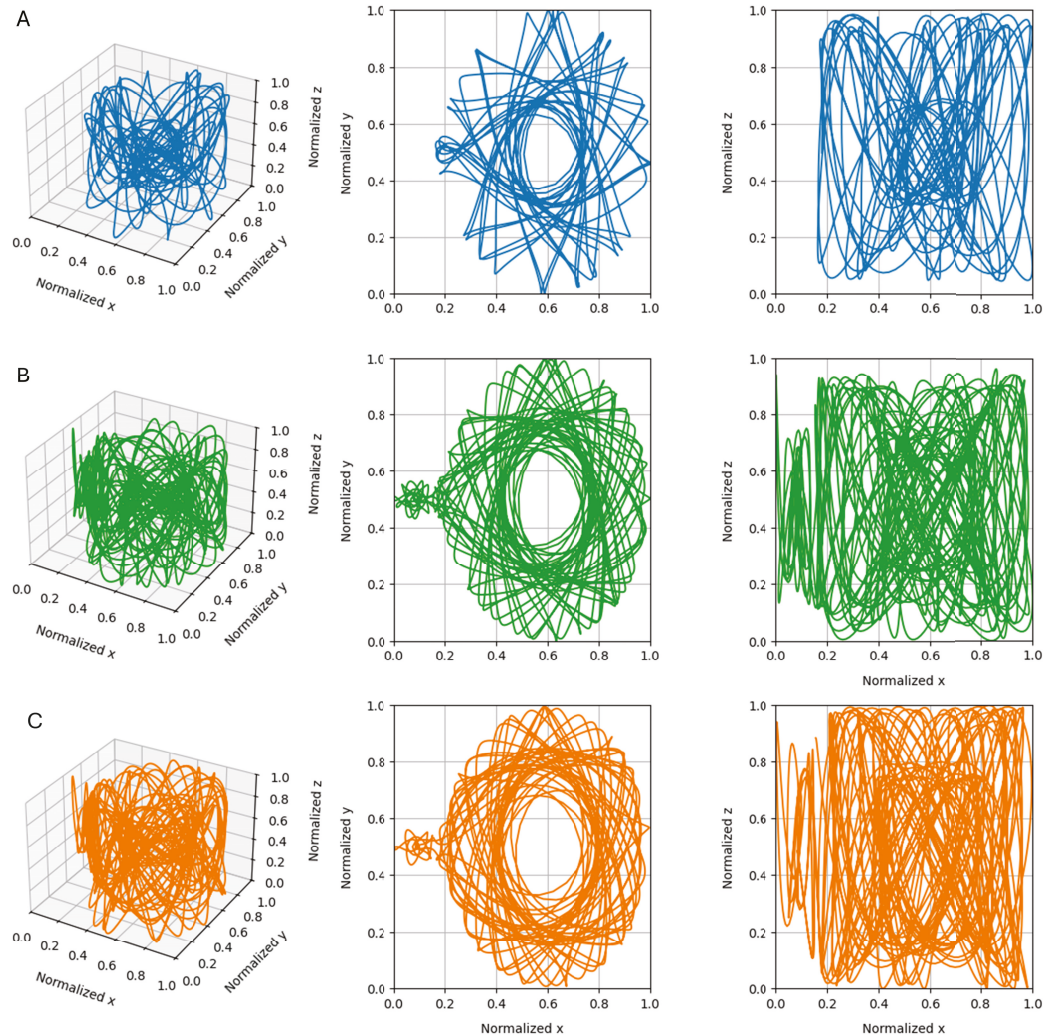


Figure 3. (A) Washout, (B) train, and (C) test subsets of the CR3BP trajectory considering $\mathbf{r} = [-0.80, 0.0, 0.0]$ and $\mathbf{v} = [0, -0.63, 0.08]$.

2.4. Experimental Protocol

Figure 4 shows the experimental protocol that was followed to obtain the results. The extraction of the connectome, the filtering of the synapses, and the construction of the connectivity matrix $\mathbf{W}$ were performed according to Section 2.1. In accordance with the existing literature [29], the parameter α was set to 0.9, or $1 - \text{timestep}$, and the input scaling was fixed at 0.7. Note that the x , y , or z time-series are presented as distinct subsets of the ESN neurons, which are selected at random before training with equal probability.

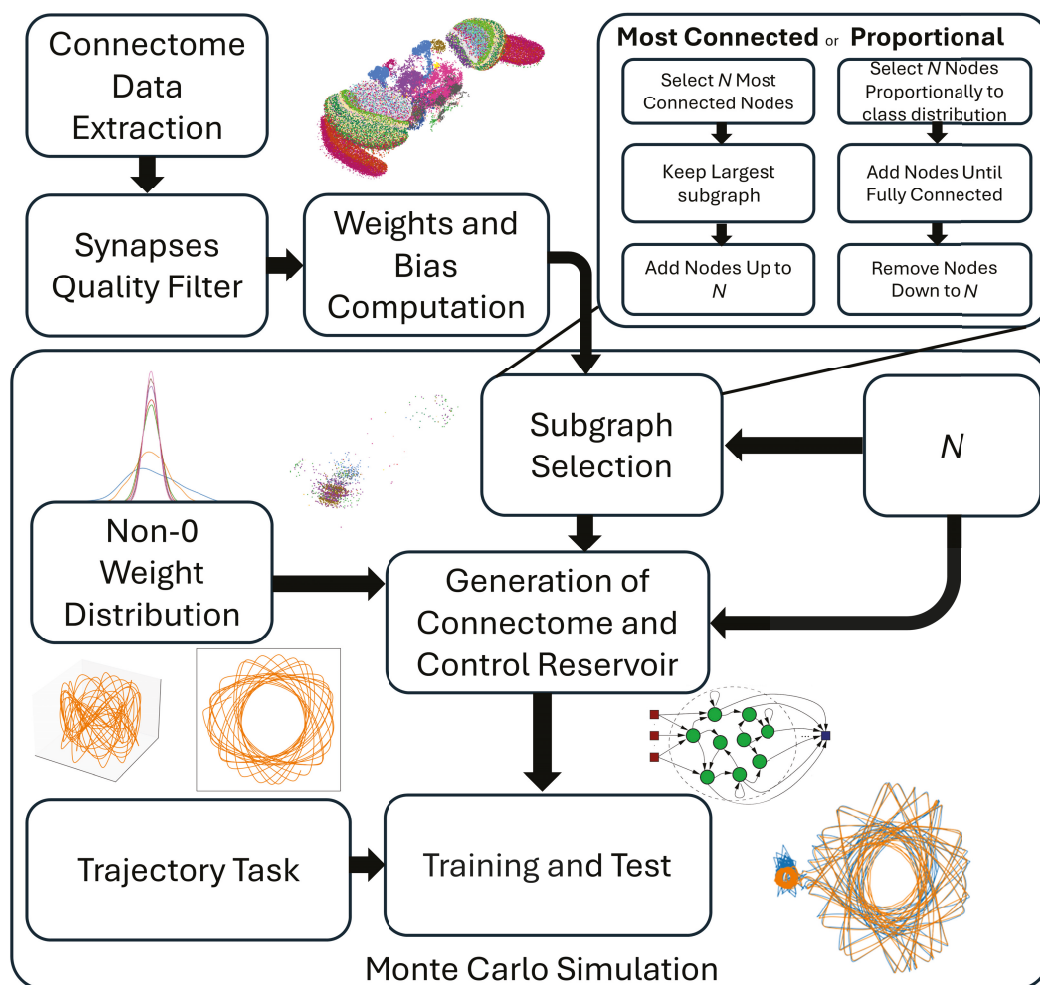


Figure 4. Flow chart of the experimental protocol. The raw data are extracted from the database and filtered, and the connectivity matrix $\mathbf{W}$ of the connectome is computed. Then, one of two possible selection criteria is used to extract a reservoir of size N , and both the connectome-inspired and the control reservoir are generated, with the same size, same input scaling, same $\mathbf{W}^{\text{in}}$, and same initial $\mathbf{W}^{\text{out}}$, prior to training. To evaluate the ESN, five trajectories are created and used to train and test the generated ESNs. A grid search is run over the values of N , β values, the random distribution of the non-zero weights, and however many timesteps into the future the ESN is trying to predict.

As a result, approximately a third of the ESN neurons receive one of the three Cartesian coordinates of the trajectory. In order to evaluate the *Drosophila* connectome as a reservoir, a comparative study was run with a control architecture through a Monte Carlo simulation. For the control architecture, both the topology and the non-zero weight distribution were generated randomly. For the topology, a connectivity matrix of the desired size was generated, with each element of the matrix having the same probability, corresponding to the desired sparsity of the reservoir, to be initialized as zero. The remaining non-zero elements represent the weight distribution of the reservoir and were generated either using a uniform or Gaussian distribution. Both the connectome-based ESN and the classical implementation were constructed with the same sparsity, reservoir size N , input scaling, input matrix $\mathbf{W}^{\text{in}}$ and initial output matrix $\mathbf{W}^{\text{out}}$ prior to training. Specifically, each neuron in the ESN receives one of the three input time-series, and all neurons are connected to the readout layer; thus, $\mathbf{W}^{\text{out}} = [\mathbf{1}; \mathbf{1}; \mathbf{1}]$, before training. This choice leads to a number of trainable parameters equal to $3 \times N$, thus between 150 for the smallest tested reservoirs and 4500 for the largest ones. The parameters that are taken into consideration for the Monte Carlo simulation are the reservoir size N , the selection criteria used to construct the

reservoir, the random distribution on the non-zero weights, the β values, and the size of the forecast horizon. Note that the weights of the matrices of both reservoirs are scaled appropriately to ensure that they have the same spectral radius of 0.99, as previously discussed. Thus, the specific absolute weights were determined through this constraint and the imposed distribution. Every combination of the tested hyperparameters was evaluated on five different trajectories, obtained by a 1% variation in the initial conditions, as shown in Figure 3, and randomly re-initialized 10 times, resulting in 50 test cases per hyperparameter combination. In total, four different architectures were compared to each other: the control case, the connectome topology with random weights, the connectome topology with the connectome weights, and the connectome weights with a random topology. This allowed us to isolate the contribution of the connectome topology from that of the synaptic weights. To evaluate the performance of the ESN, the normalized root mean squared error (nRMSE) was computed on both the training and the test set, as follows:

$$\text{nRMSE} = \frac{\sqrt{\|\mathbf{Y} - \mathbf{W}\mathbf{X}\|^2}}{\bar{\mathbf{Y}}} \quad (7)$$

where $\|\cdot\|^2$ is the squared Euclidean norm and $\bar{\mathbf{Y}}$ is the mean of $\mathbf{Y}$.

The Mann–Whitney–Wilcoxon test was used to investigate the statistical significance of the difference in performance of the different architectures [56]. To further characterize the difference in performance, the Bhattacharyya distance was used to define the distance between the nRMSE distributions obtained using two different architectures. The Bhattacharyya distance, D_B , is a measure of similarity between two probability distributions, often used to quantify the overlap between them, and can be computed as [57]

$$D_B(p, q) = -\ln\left(\sum_i^n \sqrt{p_i q_i}\right) \quad (8)$$

where p and q are datapoints belonging to the nRMSE distributions of the two tested architectures, and n is the number of datapoints, 50 in this case. This metric is then averaged across all tested reservoir sizes, N , and regularization values, β , in order to estimate the difference in performance between different architectures.

Then, an additional architecture was tested in the same conditions as the other four, keeping both the connectome topology and the connectome weights but shuffling the weights' values to obtain a random permutation of the original ones. Such an architecture allows us to investigate the synergistic relationship between topology and the distribution of weights in the connectome itself, since it retains both of them but completely changes the mapping between every synapse and its respective weight.

Lastly, a smaller experiment was performed to investigate the role of the spectral radius. The performance of the reservoirs was investigated as a function of the spectral radius in the case of $\beta = 10^{-6}$ and a forecast horizon of one timestep for different values of the reservoir size, N , selection criteria, and random distributions in the control case. Although previous studies have demonstrated higher performance closer to the edge of chaos, occurring at a spectral radius close to 1, we opted for a more thorough characterization of how the observed performance changes in our specific case for smaller values of the spectral radius.

3. Results

3.1. Selection Criteria

First, it is important to investigate which subgraph of the entire connectome is taken into consideration and how its characteristics, in terms of the nature of the selected neurons

and the topology of the subgraph itself, are affected by the considered size N . As mentioned in Section 2.1, we investigated two possible criteria: the N^{th} most connected neurons, either regardless of the classes or proportional to the total class distribution shown in Figure 1. Figure 5 shows the general results regarding the sparsity and the non-zero weight distribution for the selection criteria as a function of N .

Starting from the sparsity at $N = 50$, we can observe that the subgraph selected with the ‘most connected’ criterion has a much lower sparsity than the ‘proportional’ criterion, as expected (see Figure 5A). Moreover, the ‘most connected’ criterion leads to monotonically increasing sparsity, which is also likely. As more neurons are added to the reservoir, both selection criteria lead to increasing sparsity, with the ‘most connected’ criterion always leading to a less sparse architecture than the ‘proportional’, as a result of its class-agnostic selection.

Concerning the distribution of extracted weights, Figure 5B shows it as a function of N , whereas Figure 5C,D show the probability density function (PDF) of the extracted weights in the case of ‘most connected’ and ‘proportional’ criteria, respectively. Notably, both criteria result in bimodal distributions, with steeper peaks as N increases, confirmed by lower values of the variance. However, it is noteworthy that the ‘proportional’ criterion is characterized by the same relative magnitude of the positive and negative peaks in the PDF as the entire connectome, whereas the ‘most connected’ selects more inhibitory than excitatory synapses. Moreover, the ‘proportional’ criterion results in a larger gap in the values both for the entire connectome and for the ‘most connected’ one. This is due to the rescaling factor that is applied to achieve the same spectral radius: for a constant size N , a sparser reservoir is rescaled less to achieve the same spectral radius. Conversely, increasing the size N generally results in steeper peaks, up to the complete connectome case. According to recent comparisons for connectivity matrix extraction for connectomes [58], the extracted PDF highlights a significant amount of both positive and negative synapses, which is a fundamental requirement for achieving higher learning performance. Moreover, heavy-tailed distributions, like the one extracted from the connectome in this paper, have already been documented as better alternatives for ESNs with respect to the more traditional approaches featuring either Gaussian or uniform distributions [45,59]. However, in our results, the two criteria differ in terms of which peak is prevalent: the ‘most connected’ criterion is characterized by a higher presence of excitatory, or positive, synapses, whereas the ‘proportional’ criterion contains a relatively higher number of inhibitory, or negative, ones.

For a better insight into the selected networks, Figure 6 shows the scatterplots and the class distribution for a few of the selected reservoirs, namely both criteria and $N = 50, 100, 700, 1500$. Compared with the full connectome (see Figure 1), it is obvious that there are major differences. In the case of the ‘most connected’ criterion, $N = 50$ is shown to include a very limited number of classes, with three overwhelmingly represented: ME, LO, and bilateral classes. This trend continues at $N = 100$, but the ME class eventually overcomes the others, representing close to half of the subgraph for $N = 1500$. Moreover, as N increases, it is possible to observe a slightly larger number of different classes, but still far from the total number of classes.

Conversely, the ‘proportional’ selection criterion shows a much higher number of different classes already at $N = 50$. However, note that the distribution is not exactly proportional to the one shown in Figure 1 due to the constraint imposed of full connectivity (see Section 2.1). As N increases, it can be seen that first ME and later ME>LO start to become more and more represented. Although these two classes are the most represented in the entire connectome, they are still over-represented in the selected subgraph. This

inevitably leads to the suppression of the other classes but still maintains a more diverse distribution than the ‘most connected’ criterion.

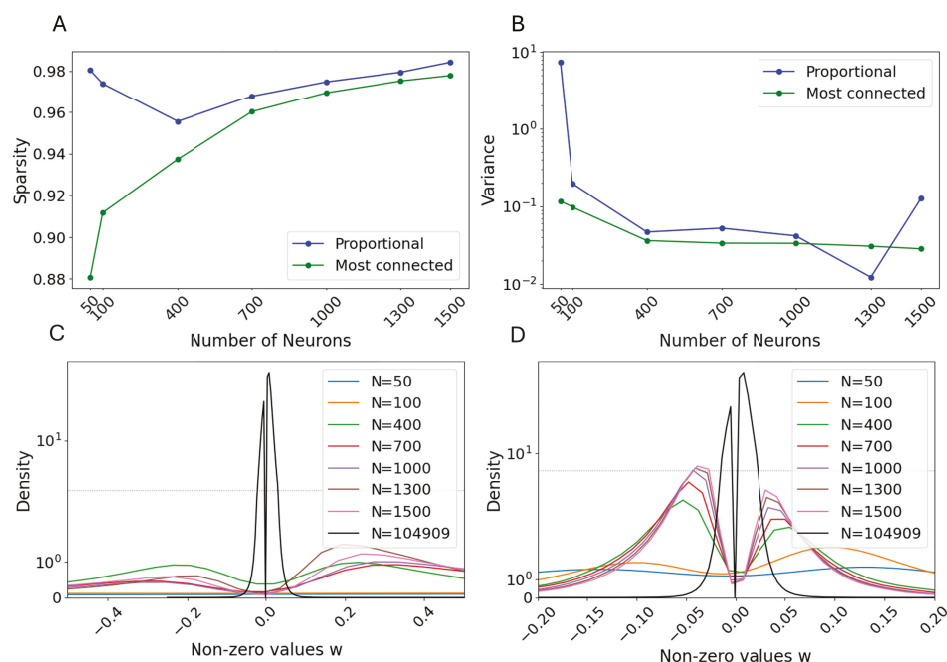


Figure 5. (A) Sparsity and (B) variance of the non-zero weights as a function of the number of neurons selected in the reservoir. The probability density function of the extracted weight in the case of (C) the ‘proportional’ criterion and (D) the ‘most connected’ criterion. For (C,D), the dotted gray line represents the threshold between the linear y scale, below the line, and the logarithmic y scale, above.

Finally, the two criteria largely differ in terms of the locations of the selected neurons within the brain. As N increases, the ‘most connected’ criterion tends to produce a more symmetrical network, adding neurons almost equally from both hemispheres. In contrast, the ‘proportional’ criterion primarily selects neurons from a single hemisphere, with higher values of N corresponding to an even more decentralized subgraph. This can be justified by the criterion trying to include all classes, even the ones that are absent in the more central cluster selected by the previous criterion, thus reaching the further layers. However, the limitation of a fixed N prevents the criterion from selecting a sufficient number of sensory neurons symmetrically, thus forcing it to primarily select from a single hemisphere.

As a last remark, the prevalence of ME and ME>LO neurons is hardly surprising. Firstly, the ME neurons represent the second processing stage of the optic lobe, which is known to process visual information, detect edges and motion, and be involved in contrast and color vision. Then, the ME>LO pathway relays information to the higher, more abstract visual areas, contributing to object recognition and image processing. These classes are not only the most abundant in the connectome of an animal that mainly deals with large quantities of visual inputs and processing, but they also represent the middle stages of this processing pipeline, being connected to both the sensory input neurons and the higher processing areas. Thus, these neurons are both the ‘most connected’ and the most over-represented when enforcing the connectedness of the constructed reservoir.

To gain a deeper insight into the topology of the selected reservoir, Figure 7 shows the degree distribution, clustering coefficient distribution, and shortest path distribution, computed as described in Section 2.1. Moreover, it shows such metrics for both selection criteria and $N = 100$ and $N = 1500$, together with a random permutation of the selected reservoirs. Random permutations are obtained by taking the selected reservoir and ran-

domly rearranging the synapses. This allows us to investigate random topologies of the same sparsity and the size of the selected networks.

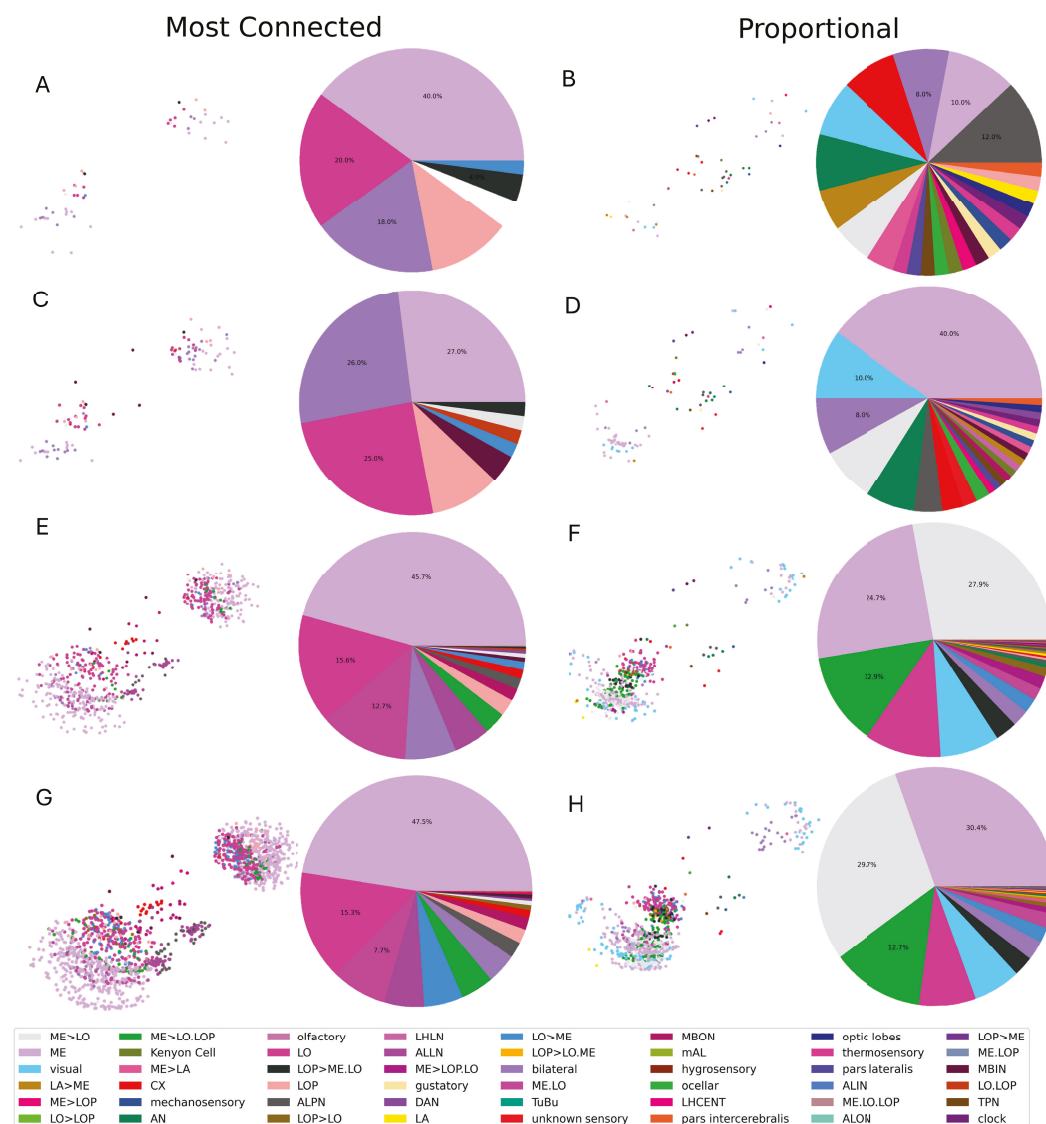


Figure 6. A 3D scatterplot of the selected reservoir and a pie chart of the class division within such a reservoir in the case of (A) the ‘most connected’ criterion and $N = 50$, (B) the ‘proportional’ criterion and $N = 50$, (C) the ‘most connected’ criterion and $N = 100$, (D) the ‘proportional’ criterion and $N = 100$, (E) the ‘most connected’ criterion and $N = 700$, (F) the ‘proportional’ criterion and $N = 700$, (G) the ‘most connected’ criterion and $N = 1500$, and (H) the ‘proportional’ criterion and $N = 1500$.

The results clearly show that the extracted reservoirs have two topological characteristics that drastically differ from their randomized counterparts: the presence of a few highly connected neurons and more degrees of separation between any two given neurons. Random topologies show a much more homogeneous configuration, with low clustering coefficients, a smaller inter-neuron shortest path on average, and a Gaussian degree distribution. Whereas these properties are expected in a random graph [60], they are radically different from what we can observe in the case of the extracted topologies, both for $N = 100$ and $N = 1500$. These topologies have a much higher clustering coefficient, as shown in Figure 7C,D, possibly due to a few super-connected neurons (see Figure 7A,B) and the presence of more separated areas, leading to the results shown in Figure 7E,F. It should also be noticed that such observations are valid for both reported sizes, indicating that the

topological properties observed here seem to be intrinsic to any subset of the *Drosophila* connectome.

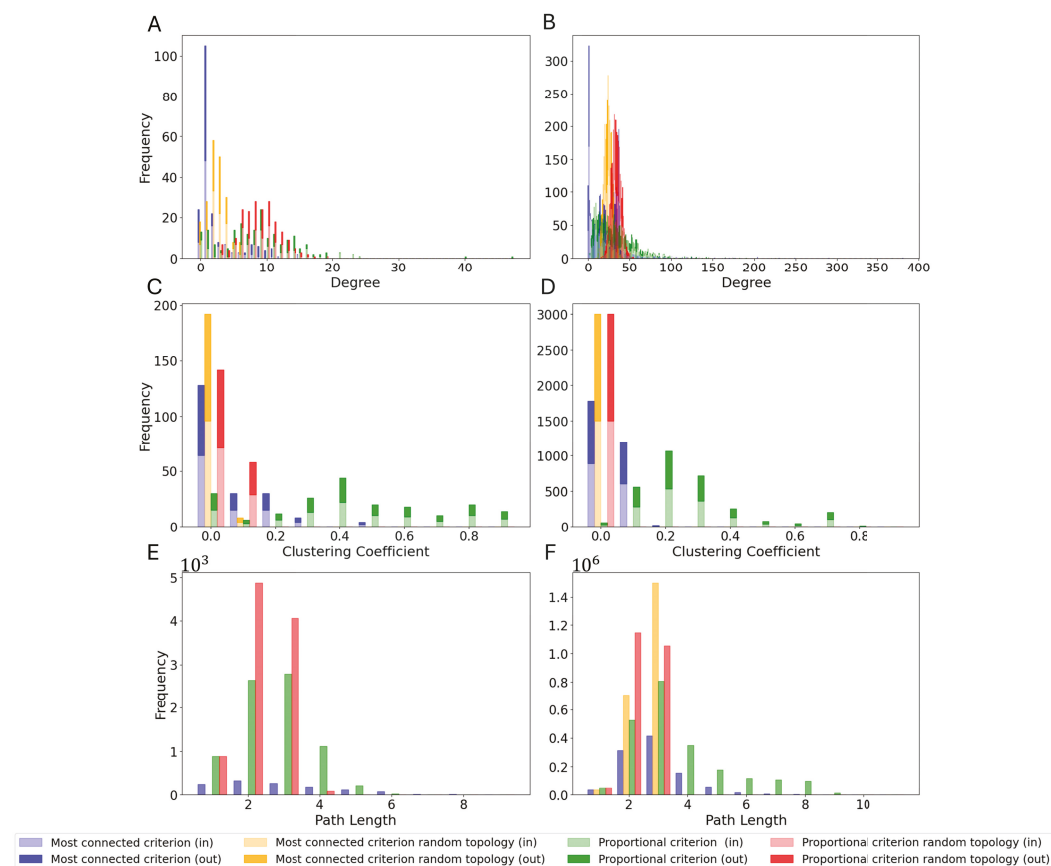


Figure 7. Topology metrics of the selected reservoirs: (A) degree distribution for $N = 100$, (B) degree distribution for $N = 1500$, (C) clustering coefficient distribution for $N = 100$, (D) clustering coefficient distribution for $N = 1500$, (E) shortest path distribution for $N = 100$, (F) shortest path distribution for $N = 1500$. Note that for the shortest path, directionality is considered while computing the path and therefore not indicated with different color intensities.

Notably, the same features can be observed in the topological characteristics of the entire connectome, as shown in Figure 8.

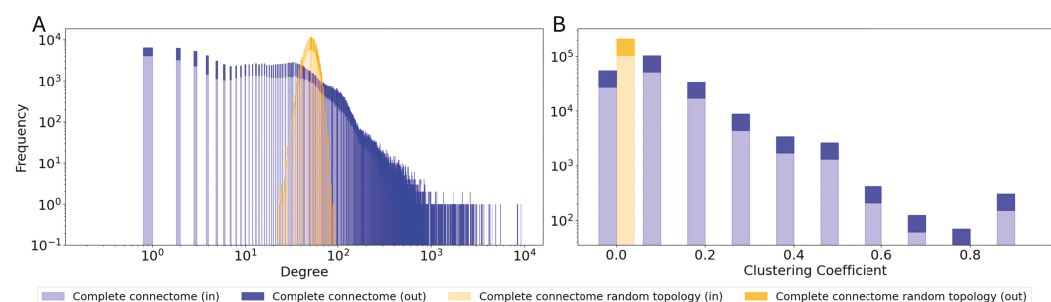


Figure 8. Topology metrics of the entire connectome as a reservoir: (A) degree distribution and (B) clustering coefficient distribution.

Unlike their random counterparts, all connectome topologies, regardless of their size, contain a much greater number of highly connected clusters. The ‘proportional’ criterion also results in significantly higher clustering coefficients than the ‘most connected’ one, closer to the highest values observed in the complete connectome. Such features have already been observed in several connectomes, including the human connectome [44].

This particular structure, known as small-world organization, has been shown to allow for local specialized processing whilst ensuring efficiency in communication and high robustness, achieved through the presence of multiple clustered fallback routes. Conversely, random topologies are characterized by a more heterogeneous connectivity matrix, with significantly lower clustering coefficients.

3.2. Single Simulation

As discussed in Section 2.2, the extracted reservoir is then included in an ESN, which is used for the prediction of multiple CR3BP trajectories. An important step in implementing an ESN is the washout, in which an initial set of points in the time-series is used as an input to the ESN in order to overwrite the initial random state of the neurons. Figure 9A,B show the state of six random neurons during the washout in the case of the classical implementation and the connectome-extracted reservoir, respectively. It can be seen that neurons have a wide range of different behaviors, while the connectome-based architecture displays slightly reduced dynamics, never reaching the boundaries of the state.

To illustrate how these factors can impact the performance of the ESN, Figure 9C–J show the performance of the ESN as a predictor of a single CR3BP trajectory. Note that these results are specific for $N = 1000$, $\beta = 10^{-6}$, and a classical implementation obtained with a uniform distribution. Figure 9C,D show that in the case of one timestep prediction, corresponding to 2.7 days, both architectures show good performance, ever so slightly in favor of the connectome-based reservoir. As expected, both architectures see a decrease in performance as the required prediction changes to 3, 7, and then 10 timesteps, which correspond to 8.1, 18.9, and 27 days, respectively. However, the classical implementation shows a more rapid and more drastic deterioration of the performance, with significantly larger loops and greater errors, at least concerning the part of the trajectory around the larger body. However, this single case cannot justify a generalization to the remaining results.

For a more detailed illustration of the origin of the observed difference in performance, Figure 10 shows the same results as in Figure 9 but tested over five different trajectories, with 10 trials per trajectory, and comparing the connectome-based reservoir to a reservoir with non-zero weights sampled from either a uniform or Gaussian distribution, as described in Section 2.4. With this methodology, both architectures are re-initialized for each trial: in the case of the classic implementation, all architecture parameters are randomized, including topology, input connections, synaptic weights, and neuronal biases. Conversely, the connectome-based architecture maintains the same topology and synaptic weights, and only the input connections and biases are randomized. Note that the connectivity matrices of all cases are created with the same sparsity, which is imposed by the topology of the selected connectome subgraph, and they are rescaled to have the same spectral radius, which is restricted to be less than 1 in order to satisfy the ESP, as indicated in the literature [29].

It is clear from the plots that the classic control reservoir tends to overfit the training, achieving a much lower nRMSE when tested on the training set. Conversely, the connectome-based reservoir shows a higher error on the training set but a consistently lower error on the test set. However, both reservoirs show signs of overfitting. This can be explained by the fully connected readout, meaning that all of the reservoir's neurons are connected to the ridge regression layers. This results in a large number of trainable parameters, which can cause overfitting, especially if paired with lower values of the regularization parameter β .

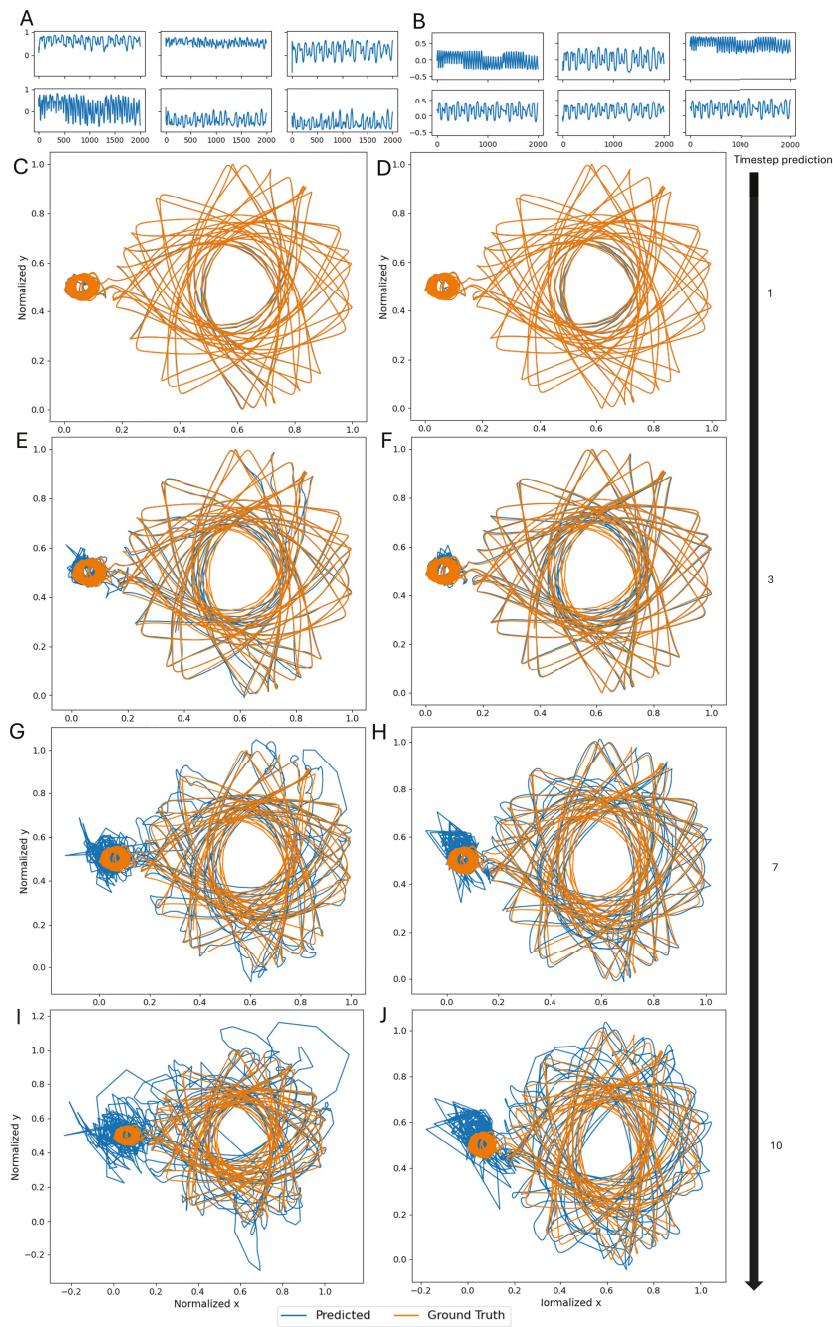


Figure 9. The neuronal activation state of six random neurons during the washout in the case of (A) a classical reservoir with a uniform non-zero weight distribution and (B) the reservoir extracted from the connectome. An example of the performance of the ESN on a single test trajectory in the case of $N = 1000$, $\beta = 10^{-6}$, and a classical implementation obtained with a uniform distribution in the case of (C) a classical and (D) connectome-based reservoir and a 1-timestep prediction; (E) a classical and (F) connectome-based reservoir and a 3-timestep prediction; (G) a classical and (H) connectome-based reservoir and a 7-timestep prediction; and (I) a classical and (J) connectome-based reservoir and a 10-timestep prediction.

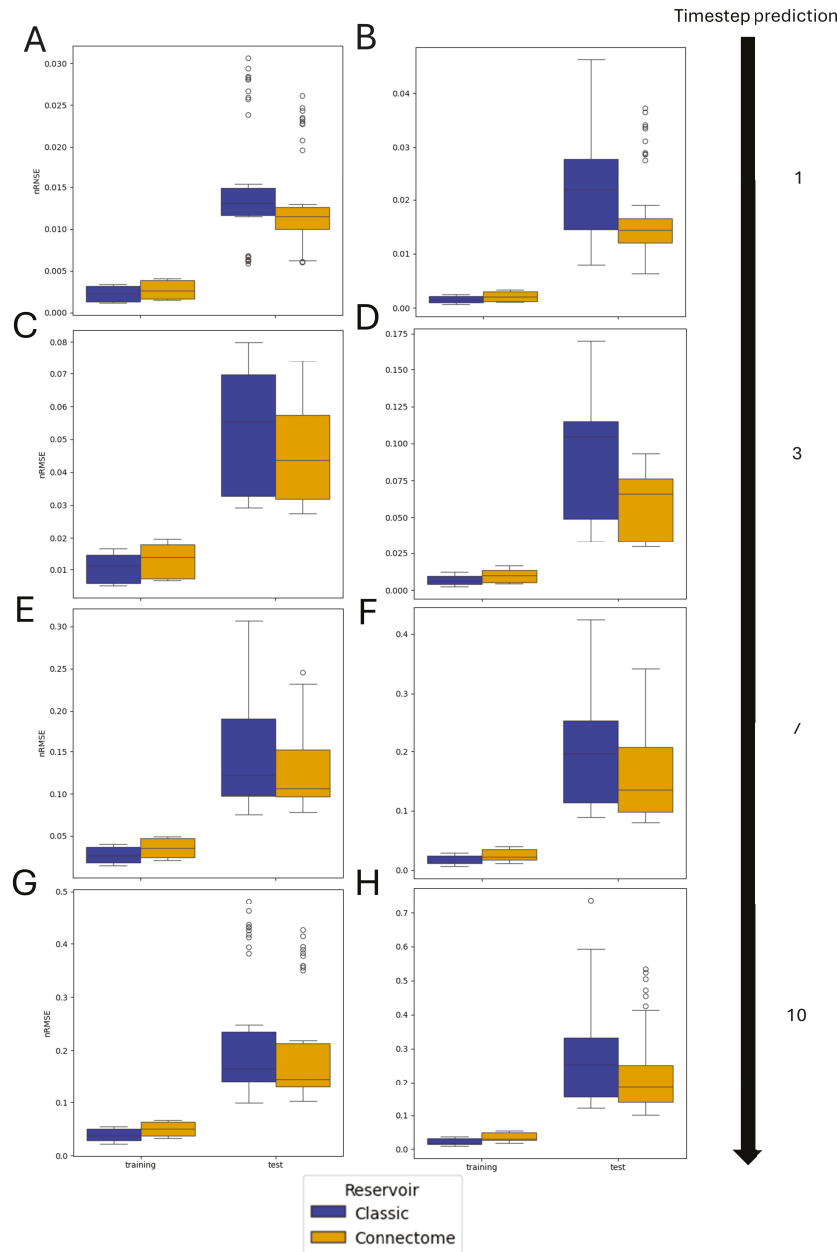


Figure 10. Comparative results of the ESN performance of the training and test sets in the case of $N = 1000$ and $\beta = 10^{-6}$. Connectome and classic refer to the connectome-based and random reservoirs, respectively. The selection criterion used for the extraction of neurons for the connectome-based ESN is the ‘most connected’ criterion. Simulations were performed for the following cases: (A) Gaussian and (B) uniform non-zero weight distribution for the classic reservoir in the case of 1-timestep prediction, (C) Gaussian and (D) uniform non-zero weight distribution for the classic reservoir in the case of 3-timestep prediction, (E) Gaussian and (F) uniform non-zero weight distribution for the classic reservoir in the case of 7-timestep prediction, and (G) Gaussian and (H) uniform non-zero weight distribution for the classic reservoir in the case of 10-timestep prediction.

3.3. Monte Carlo Simulations

To fully characterize the connectome-based reservoir both in terms of its topology and the distribution of non-zero weights, a comparative study was performed according to Section 2.4. It is important to note, as previously discussed, that the hyperparameters involved in this investigation are the size of the selected reservoir N , the type of distribution used for the selection of random variables, either uniform or Gaussian, the prediction horizon, and the selection criterion used to extract the connectome-based reservoir from

the full connectome. All other hyperparameters were kept constant across the tested cases, and every combination was tested 10 times per trajectory over five different trajectories. The four different architectures that are part of this comparative study are as follows:

- Random topology and random weight distribution, which is the standard way to create ESNs, and represents the control group;
- Connectome-based topology and extracted weight distribution, utilizing all the data extracted from the *Drosophila* connectome;
- Connectome-based topology and random weight distribution to solely isolate the contribution of the topology of the connectome;
- Random topology and extracted weight distribution to solely isolate the contribution of the weight distribution of the connectome.

Figures 11 and 12 show the results of the Monte Carlo simulation for the ‘most connected’ and ‘proportional’ selection criteria, respectively.

Note that the results at $\beta = 10^{-6}$ confirm the insights given by the previous observations (see Figure 10), showing a decrease in performance for the classical implementation with respect to the other three. Moreover, it can be seen that such a difference increases as N increases. As expected, as the prediction horizon increases, there is a drop in performance (as shown in Figure 9). However, for all tested prediction horizons, the architecture with both a connectome topology and connectome weight distribution does not show a decrease in performance for any tested N . In the case of the hybrid architectures, with a connectome topology and random weights or vice versa, the results show that their performance lies between that of the connectome-inspired architecture and the control one. This seems to indicate that both the non-zero weight distribution and the topology play an effective role in counteracting overfitting, given the improvement achieved with just the connectome topology or with its weight distribution. Moreover, the relative performance of the four tested cases is consistent across different conditions, with the random topology and connectome-extracted weights always performing better than the connectome topology and random weights, but worse than the fully biomimetic architecture, for large N . This result confirms that both the distribution of weights and the topology play a role in the increase in performance, but the effect of the distribution of weights is stronger.

Next, in the cases with lower regularization (i.e., $\beta = 10^{-9}$), it can be observed that the relative performance of all tested architectures is largely unchanged. Nevertheless, even the connectome topology and connectome weight case starts to show overfitting for larger N . Noticeably, this phenomenon appears earlier for short prediction horizons (Figures 11A–D and 12A–D) than for longer horizons (Figures 11E–H and 12E–H). This behavior can be explained by the lower regularization, which naturally makes the ESN more prone to overfitting. Moreover, these results indicate that ESNs with a random Gaussian weight distribution are more resilient to overfitting compared to ones initialized with a uniform distribution, with smaller differences in performance between the four tested architectures as N increases. Similarly to the previous case, the distribution of weights taken from the connectome shows a stronger effect on the error reduction than the topology when considered in isolation, and the fully biomimetic architecture, despite starting to show a decrease in performance as N increases, is still the most resilient.

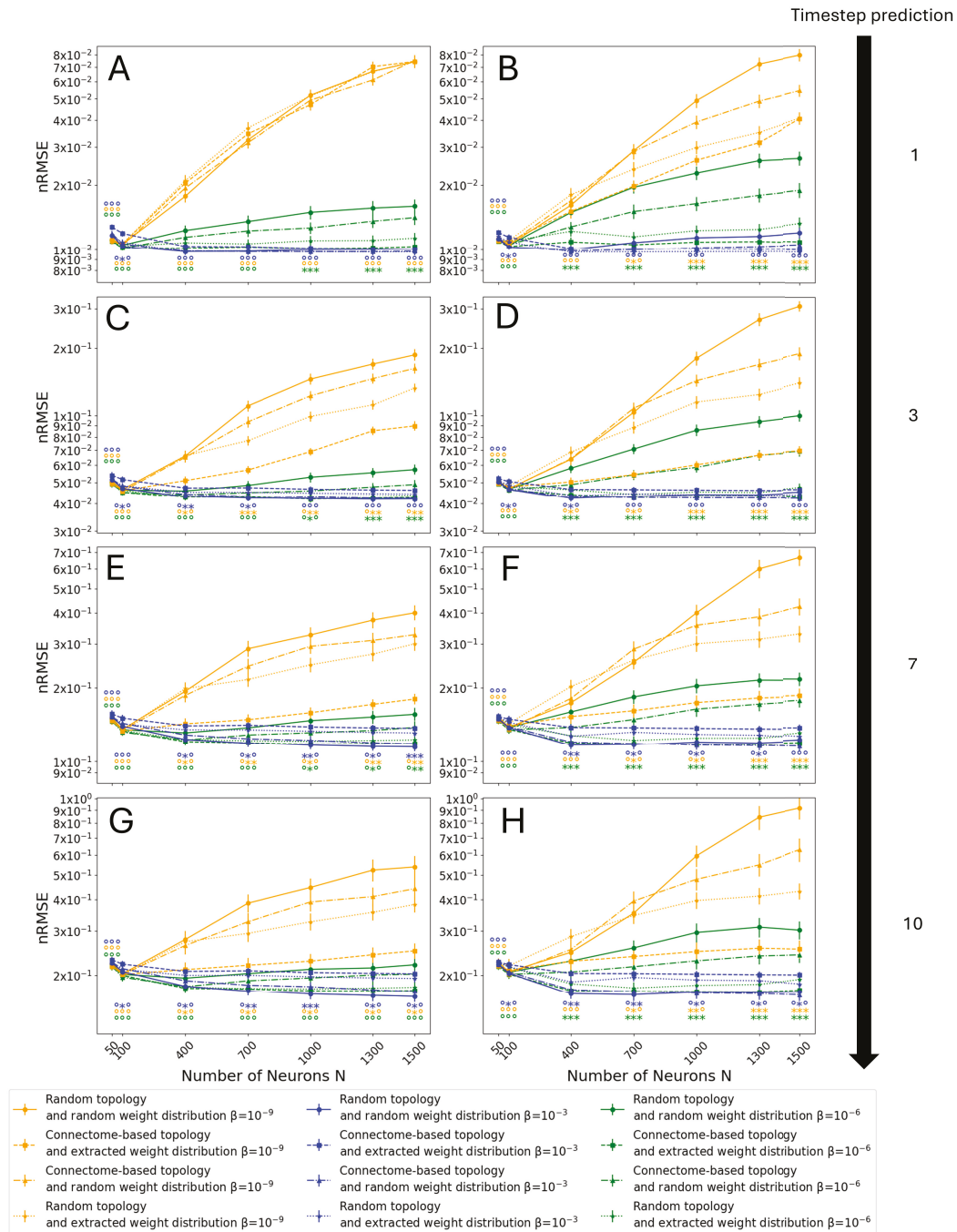


Figure 11. Results on the test set as a function of reservoir size, N , and normalization factor, β , using the 'most connected' selection criterion. Simulations were performed for the following cases: (A) Gaussian and (B) uniform non-zero weight distribution for the classic reservoir with 1-timestep prediction, (C) Gaussian and (D) uniform non-zero weight distribution for the classic reservoir with 3-timestep prediction, (E) Gaussian and (F) uniform non-zero weight distribution for the classic reservoir with 7-timestep prediction, and (G) Gaussian and (H) uniform non-zero weight distribution for the classic reservoir with 10-timestep prediction. Error bars display the mean's standard deviation. Statistically significant differences ($p < 0.05$) under the Mann–Whitney–Wilcoxon test are indicated by the marker *, and no significance is indicated by the marker o as follows: the control case of random topology and random weight distribution was tested against the connectome-based topology and random weight distribution (left marker), the connectome-based topology and extracted weight distribution (central marker), and the random topology and extracted weight distribution (right marker).

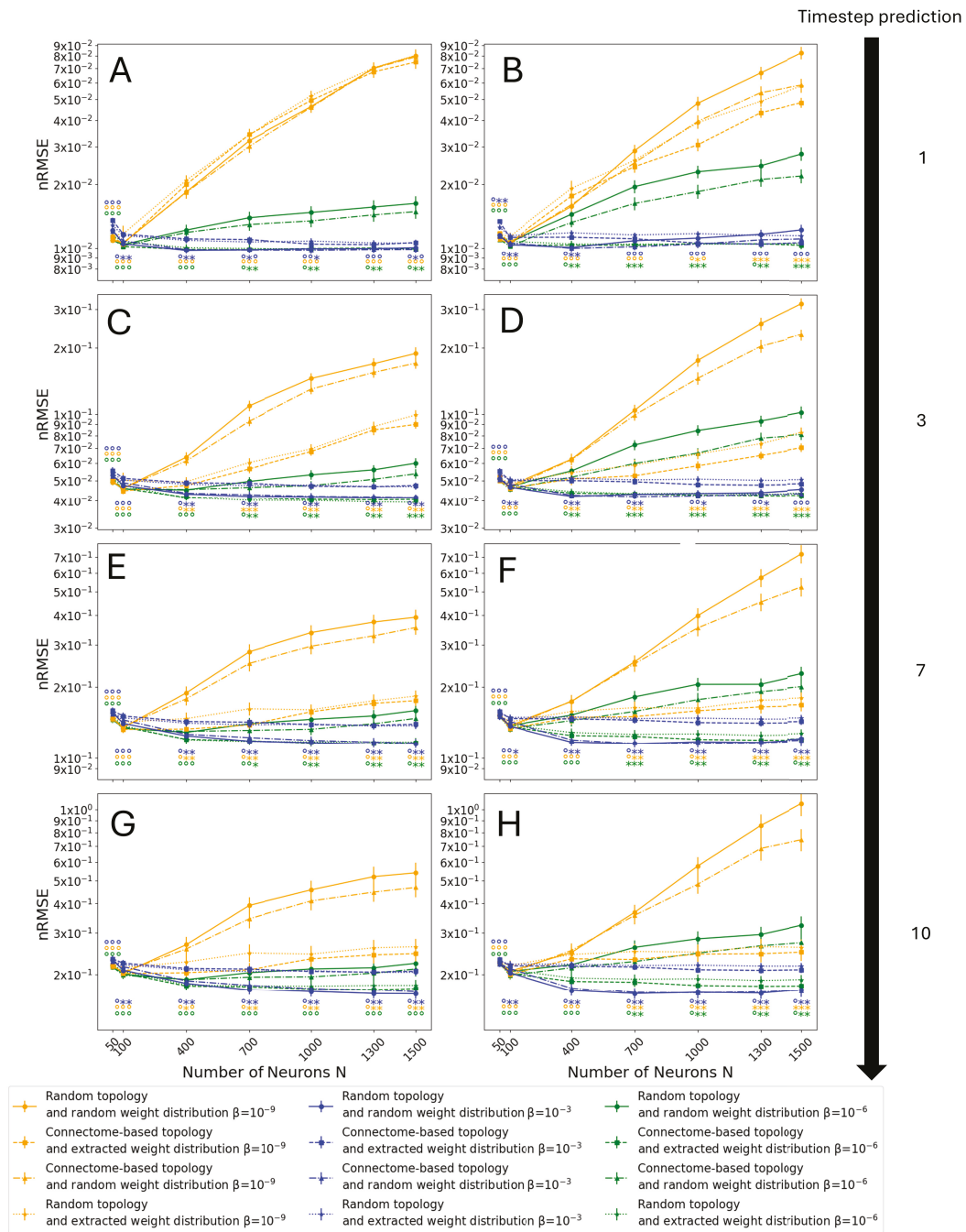


Figure 12. Results on the test set as a function of reservoir size, N , and normalization factor, β , using the ‘proportional’ selection criterion. Simulations were performed for the following cases: (A) Gaussian and (B) uniform non-zero weight distribution for the classic reservoir with 1-timestep prediction, (C) Gaussian and (D) uniform non-zero weight distribution for the classic reservoir with 3-timestep prediction, (E) Gaussian and (F) uniform non-zero weight distribution for the classic reservoir with 7-timestep prediction, and (G) Gaussian and (H) uniform non-zero weight distribution for the classic reservoir with 10-timestep prediction. Error bars display the mean’s standard deviation. Statistically significant differences ($p < 0.05$) under the Mann–Whitney–Wilcoxon test are indicated by the marker *, and no significance is indicated by the marker o as follows: the control case of a random topology and random weight distribution was tested against the connectome-based topology and random weight distribution (left marker), the connectome-based topology and extracted weight distribution (central marker), and the random topology and extracted weight distribution (right marker).

Conversely, the ESN behavior at $\beta = 10^{-3}$ shows drastically different trends. On one hand, there is no decrease in performance as N , and thus the number of trained degrees of freedom, increases. This result reinforces the conclusion that overfitting is the origin of the decrease in performance since higher β values specifically counteract overfitting. On the other hand, the connectome topology and connectome weight architecture shows the highest error almost consistently in different conditions. However, the connectome topology with random weight initialization still closely matches the performance of the classical implementation, and larger reservoirs with a uniform weight distribution eventually outperform the control architecture with random topology and random weights.

Comparing the corresponding subfigures in Figures 11 and 12, the results are extremely similar, despite the differences in the topology of the selected connectome subgraphs that constitute the reservoirs (see Section 2.1). The most noticeable difference between the two Monte Carlo simulations is that the results corresponding to the architecture with the connectome topology and random weights are closer to those for the control architecture in the case of ‘proportional’ selection. Moreover, this combination of topology and weight distribution is closer in performance to the control case than the architecture with connectome weights and a random topology, which in turn is closer to the complete connectome-inspired architecture. This indicates that the contribution of the topology towards the mitigation of overfitting is present but small, and the observed bimodal distribution of non-zero weights (see Figure 5) plays a bigger role.

To further investigate the synergistic roles of synaptic weights and topology, the case of a connectome topology and shuffled connectome weights was also tested. As discussed in Section 2.4, this particular architecture has both the same non-zero weight distribution and the same topology as the fully biomimetic connectome-inspired one but does not have the same mapping between the synapses and their respective weights. The performance of this architecture is statistically equivalent to the previously tested case of connectome weights and a random topology, as the Mann–Whitney–Wilcoxon test shows a p -value > 0.05 for all tested combinations of N and β . Moreover, the Bhattacharyya distance between the nRMSE distribution of the two architectures was computed and averaged over all reservoir sizes, N , and all regularization values, β , resulting in $3.2 \times 10^{-3} \pm 2 \times 10^{-3}$ and $1.6 \times 10^{-3} \pm 1 \times 10^{-3}$ in the case of the ‘most connected’ and the ‘proportional’ selection criterion, respectively. These values indicate that shuffling the weights extracted from the connectome has the same effect on the performance as randomizing the topology of the reservoir. This indicates not only that both the connectome weight distribution and its topology play a role in the observed overfitting resilience but also that they are strongly bound to each other. In the case of the fully biomimetic connectome-inspired design, the position of every synaptic weight within the topology is key to achieving the observed properties. While this was outside the scope of this study due to the sheer size of the networks being explored, one potential avenue for future study would be to investigate how the performance of the ESN declines as progressively larger portions of the weights are shuffled.

As discussed in Section 2.1, a spectral radius of 0.99 was selected in accordance with previous literature on ESNs and biomimetic ESNs. However, in order to further characterize the difference in performance between the connectome-based reservoir and the control architecture, a set of simulations with lower spectral radii, such as 0.75, 0.5, and 0.25, were performed. These simulations follow the same experimental protocol as the previous ones, but they only feature reservoirs with regularization $\beta = 10^{-6}$ and a forecast horizon of one timestep. Figure 13 showcases the difference in nRMSE between the control case and connectome-based reservoirs, respectively, as a function of reservoir size N for different selection criteria and random distributions.

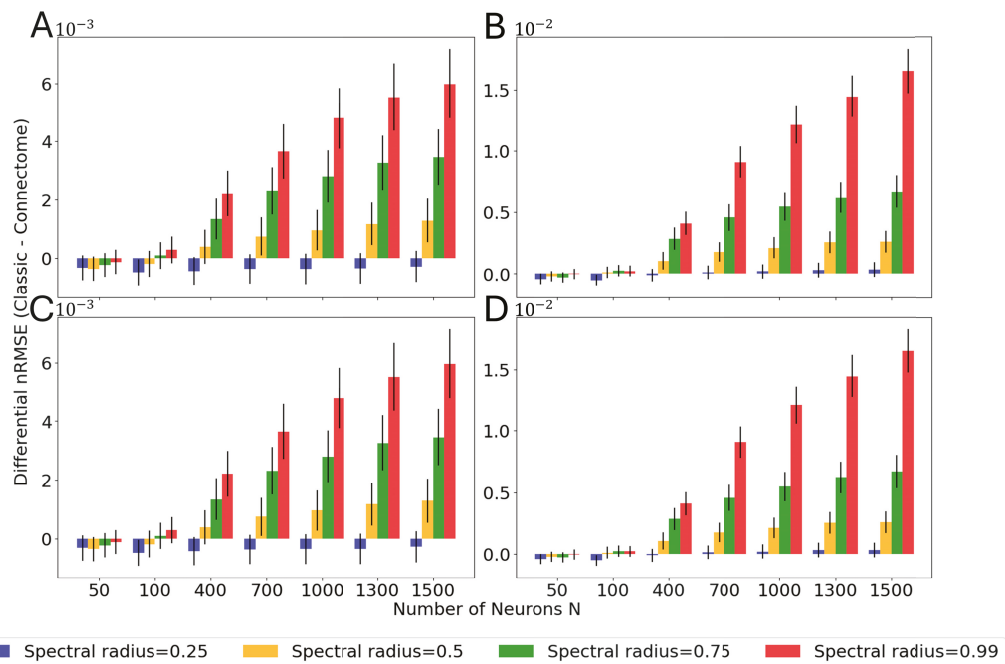


Figure 13. Differential nRMSE between classical and connectome nRMSE, respectively, for $\beta = 10^{-6}$ and one-timestep forecast horizon as a function of reservoir size, N , in the case of (A) a Gaussian and (B) uniform non-zero weight distribution for the classic reservoir and ‘most connected’ selection criterion for the connectome-based reservoir and (C) a Gaussian and (D) uniform non-zero weight distribution for the classic reservoir and ‘proportional’ selection criterion for the connectome-based reservoir.

It is noteworthy that, in agreement with previous studies [44,45], connectome-based architectures perform better at high spectral radii. Among the tested cases, while the biomimetic reservoirs always outperform the classical ones for a spectral radius of 0.99, they are equivalent, and even underperform, for lower ones, especially for smaller sizes. In the case of a spectral radius of 0.25, the classical ESN consistently performs better than the connectome-based ESN, with very few exceptions. In summary, a spectral radius of 0.99 yields better performance than at least 0.25, 0.5, and 0.75 in both models.

All investigated conditions seem to lead to the conclusion that both synaptic weights and the reservoir topology play a role, albeit of different magnitude, in preventing overfitting as N increases within a certain range of the regularization parameter, β . This phenomenon can be overshadowed by large β values, and its effectiveness seems to be lower for very low values of β . However, the effect of $\beta < 10^{-9}$ has not been investigated as the performance of the ESNs at $\beta = 10^{-9}$ already shows high nRMSE for relatively small N .

3.4. Entire Connectome Simulations

The results investigated up to this section were all obtained using a relatively small reservoir, up to $N = 1500$. Whilst the tested sizes are coherent with state-of-the-art reservoir computing, they are still several orders of magnitude smaller than the size of the actual brain. Since the purpose of this work is to investigate the connectome as a reservoir, a final study using the entire connectome was performed. As mentioned in Section 2.1, the entire connectome, after quality filtering, consists of 104,909 neurons, forming a single connected graph. Therefore, it is not computationally possible to keep all neurons connected to the readout layer, namely $\mathbf{W}^{\text{out}} = [1; 1; 1]$, as the single-shot training needed by ridge regression (see Equation (6)) relies on the inversion of the matrix $\mathbf{X}\mathbf{X}^T + \beta\mathbf{I}$, which is an $n \times n$ matrix, where n is the number of non-zero elements in $\mathbf{W}^{\text{out}}$. As a solution, we

limited both the neurons connected to the output and the ones receiving the data from the input according to Table 2 and based on the *Drosophila*'s neurophysiology.

Table 2. Classification of neuron classes into input, intermediate, and output categories. The input neurons receive the input signal, whereas the output neurons are connected to the readout.

Type	Neuron Classes
Input	olfactory, visual, mechanosensory, hygrosensory, thermosensory, gustatory, ocellar, unknown sensory
Intermediate	CX, ALPN, LO, bilateral, ME, ME>LOP, ME>LO, LO>LOP, ME>LA, LA>ME, ME>LO.LOP, ALLN, LOP>ME.LO, LOP>LO.ME, LA, AN, ALIN, mAL, LHLN, ME.LO, ME.LO.LOP, LO>ME, optic lobes, ME.LOP, TPN
Output	MBON, DAN, LHCENT, clock, pars intercerebralis, pars lateralis, Kenyon Cell, ALON, LOP>ME, LOP>LO.ME, LOP>LO, LOP, TuBu

Neurons belonging to the input classes receive the input signal, whereas the ones belonging to an output class are connected to the readout layer. The proposed rough classification is loosely based on the *Drosophila*'s neurophysiology [61], and it is used solely to reduce the computational complexity of the training process. Moreover, only for this part of the investigation, each trajectory was tested 3 times instead of 10 to compensate for the large computational time required due to the dimensionality of the matrices involved. Similarly, only a single-timestep prediction was attempted. Figure 14 shows a comparison between ESN architectures with a random topology and a random weight distribution on one hand and a connectome-based topology and an extracted weight distribution on the other.

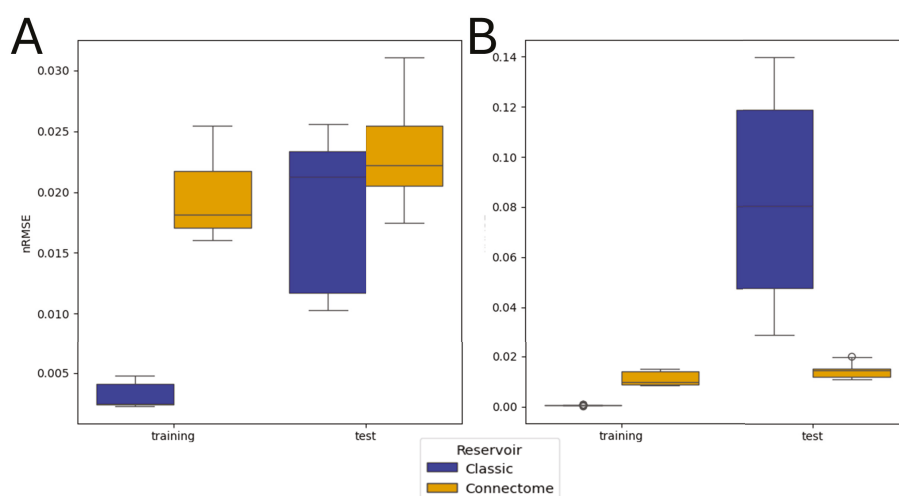


Figure 14. Comparative results of the ESN performance of the training and test sets in the case of the whole connectome predicting one timestep into the future. The control architecture uses a uniform distribution of non-zero weights. Connectome and classic refer to the connectome-based and manually constructed reservoirs, respectively. This study was performed in the following conditions: (A) $\beta = 10^{-3}$ and (B) $\beta = 10^{-6}$.

The results confirm the trends that were already observed in the Monte Carlo simulations. On one hand, when $\beta = 10^{-3}$, it can be seen that the control architecture outperforms the connectome-based one on both the training and the test sets. On the other hand, the results for $\beta = 10^{-6}$ are drastically different, demonstrating the connectome-based architecture's resilience to overfitting. The connectome-based architecture achieves this despite the large number of trainable parameters, just over 22×10^3 , three times the number of all neurons belonging to the output classes, which increases the chance of overfitting.

An interesting result is that the connectome-based architecture with $\beta = 10^{-6}$ not only outperforms the control case for the same β but also outperforms both architectures when $\beta = 10^{-3}$, with an nRMSE $< 2\%$. Once more, the connectome-based reservoir shows much higher resilience to overfitting, as indicated by the small difference in performance between the training and test phases.

4. Conclusions

In this work, the possibility of using the scanned connectome of a *Drosophila* as a reservoir for an ESN is investigated, and its performance as a time-series predictor is characterized through a comparative study. As per state-of-the-art guidelines [29], the proposed and control architectures are compared while enforcing the same spectral radius, reservoir size, number of neurons that receive the input, input scaling, and number of neurons that are connected to the readout layer. The two main features of the architecture that are investigated are the topology of the reservoir itself and the distribution of non-zero weights. In the control case, the topology is randomized, and the non-zero weight distribution is either Gaussian or uniform. To further isolate the role of the topology, hybrid architectures are also added to the study: one with the topology based on the connectome and a Gaussian or uniform distribution of non-zero weights and one with a randomized topology and the weights that have been extracted from the connectome.

In order to consider reservoir sizes similar to those used in previous literature, two possible selection criteria are adopted. Noticeably, the two criteria lead to different neurons being selected to participate in the connectome-based ESN, where the neurons differ in terms of their biological class and their position within the brain. Although the ‘most connected’ criterion results in a more symmetric selection while the ‘proportional’ criterion primarily selects neurons from the right hemisphere, the results show that the performance is not affected by the selection criterion. However, regardless of the selection criterion, all connectome-based reservoir topologies show a high degree of clustering. We observe, in line with previous literature, that highly connected neurons provide robustness, and shortcuts between anatomically distant brain regions ensure fast communication. Thus, we hypothesize that the increased robustness of such topologies with respect to the more heterogeneous ones of the control cases can lead to the observed higher resilience to overfitting. Concerning the extracted synaptic weights, the reservoirs are characterized by a heavy-tailed bimodal distribution. This is also consistent with previous studies on both traditional and connectome-inspired ESNs and has been shown to lead to increased learning performance with respect to other more traditional distributions, such as uniform or Gaussian. All in all, our findings support a possible generalization of the results of this study: despite working on significantly smaller reservoirs than the size of the entire brain, the results are consistent across different connectome regions, indicating that the investigated properties are not localized but rather extend across the connectome.

Regarding the main results of the study, Monte Carlo simulations are run to span over a wide range of parameters, including the regularization of the readout layer, the considered ESN size, the size of the prediction horizon, and the random distribution governing the control architecture. The outcome indicates that the connectome-based reservoir is significantly more resilient to overfitting than a standard implementation. Weaker readout normalization, which potentially allows for more pronounced overfitting, was found to have a much smaller effect on the connectome-based ESN than the control one. Specifically, the fully biomimetic ESN, with both the topology and weights extracted from the connectome, was significantly less prone to overfitting with increasing reservoir size compared to the other studied architectures. As previously discussed, we believe that the increased robustness due to the higher clustering and the small-world topology,

particularly in the case of the ‘proportional’ selection criterion, and the higher learning ability of bimodal heavy-tailed distributions for synaptic weights are the main factors behind the observed behavior. The case in which the connectome-based reservoir shows the worst performance is for high values of the regularization parameter, in which case overfitting is much less likely to happen in the first place.

Additionally, the performance of the ESN with a connectome topology and random non-zero weights is almost always within the range defined by the performance of the other two architectures, indicating that both the topology and extracted weights play a role in the observed resilience to overfitting. Notably, our investigation revealed that the unique mapping between the weight distribution and the topology of the connectome is key to further augmenting this property, since a permutation of the synaptic weights results in a performance that is statistically identical to that of a connectome with a completely random topology and weights. Thus, anatomical features, such as the small-world organization of the topology of the reservoir, and physiological ones, such as the distribution of synaptic weights, cannot be treated independently. In other words, both the presence of clusters and the relative weights of all redundant pathways within them are crucial to achieving a higher resilience to overfitting. The exception is the case of high regularization, $\beta = 10^{-3}$, in which the hybrid architecture occasionally shows the best performance for higher reservoir sizes, such as 1300 and 1500.

An investigation was also conducted using the entire connectome as a reservoir. Given the large number of trainable parameters, overfitting is to be expected, and such is the case for the control architecture. Conversely, the connectome-based architecture is characterized by minimal overfitting, with similar errors on the training and test sets. Moreover, the connectome-based ESN shows a slightly better performance with lower regularization, with an error at $\beta = 10^{-6}$ that is also lower than that of both tested architectures at $\beta = 10^{-3}$. This further supports the link between the properties of the reservoir and its resilience to overfitting, especially for larger reservoirs.

This study is an attempt to understand the computing capabilities of biological brains within the framework of time-series prediction enabled by AI and neural networks. We acknowledge the limitations of the investigation regarding the tested conditions, application tasks, and the size of the study itself. Thus, we encourage future work aimed at the expansion and generalization of the results hereby showcased, starting from the extension of the complete connectome as a reservoir beyond the specific task of time-series prediction. Additional future work can focus on alternative biologically plausible selection criteria in order to unveil additional properties that might be strongly related to a particular subsection of the connectome. Lastly, a study on other animals’ full connectomes, as soon as complete scans are available, is clearly the key to understanding if the observed resilience to overfitting is a property of biological brains in general, as we are hypothesizing, or if it is specifically a property of the brain of *Drosophila*. Lastly, a possible future research direction could be the physical implementation of biomimetic connectome-based reservoirs, ranging from more abstract artificial implementations of neuromorphic brain-like networks, such as clustered nanowires [62,63], to the more drastic approach of using real biological brains or neurons, exploiting organoid intelligence [64].

In summary, this study has demonstrated that, in the framework of reservoir computing for time-series prediction, both the topology and the synaptic weights of a real biological brain have an edge with respect to state-of-the-art implementation with respect to overfitting. Such results unveil a peculiar property of the brains of *Drosophila* and potentially other animals. We conjecture that, considering the complex nature of animals and the vast range of tasks that their bodies need to accomplish at any given time, overfitting to a specific task would lead to a drop in other functions.

Author Contributions: Conceptualization, all authors; methodology, L.C., A.H. and D.D.; software, L.C. and D.I.; validation, L.C.; formal analysis, L.C.; investigation, L.C.; resources, L.C. and D.I.; data curation, L.C.; writing—original draft preparation, L.C.; writing—review and editing, L.C., A.H., D.D., Z.F.H. and D.I.; visualization, L.C.; supervision, D.I.; project administration, D.I.; funding acquisition, D.I. All authors have read and agreed to the published version of the manuscript.

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Article

Model Predictive Control with Optimal Modelling for Pneumatic Artificial Muscle in Rehabilitation Robotics: Confirmation of Validity Though Preliminary Testing

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Abstract: This paper presents a model predictive controller (MPC) based on dynamic models generated using the Particle Swarm Optimisation method for accurate motion control of a pneumatic artificial muscle (PAM) for application in rehabilitation robotics. The physical compliance and lightweight nature of PAMs make them desirable for use in the field but also introduce nonlinear dynamic properties which are difficult to accurately model and control. As well as the MPC, three other control systems were examined for a comparative study: a particle-swarm optimised proportional-integral-derivative controller (PSO-PID), an iterative learning controller (ILC), and classical PID control. A series of different waveforms were used as setpoints for each controller, including addition of external loading and simulated disturbance, for a system consisting of a single PAM. Based on the displacement error measured for each experiment, the PID controller performed worst with the largest error values and an issue with oscillating about the setpoint. PSO-PID performed better but still poorly compared with the other intelligent controllers, as well as still exhibiting oscillation, which is undesirable in any human–robot interaction as it can heavily impact the comfort and safety of the system. ILC performed well with rapid convergence to steady-state and low-error values, as well as mitigation of loads and disturbance; however, it performed poorly under changing frequency of input. MPC generally performed the best of the controllers tested here, with the lowest error values and a rapid response to changes in setpoint, as well as no required learning period due to the predictive algorithm.

Keywords: pneumatic artificial muscle; control systems; machine intelligence; rehabilitation robotics

1. Introduction

Physical therapy for the restoration of motion in physically and neurologically injured limbs is an incredibly important procedure for the millions of people suffering limited motion worldwide, but with the increasingly limited healthcare services available this puts incredible strain on therapists and healthcare workers. According to the World Stroke Organization, between the years 1990 and 2019 there were more than 12.2 million new stroke cases each year, and by 2019 over 101 million people lived post-stroke [1]. With 20–25% of stroke survivors never being able to walk without assistance, and 65% unable to use their affected hands normally [2], this issue is widespread and affects a great number of people every year. In an attempt to reduce the strain on healthcare services and provide the needed care to patients, rehabilitation robotics is an open research field aiming to develop and produce robots capable of giving patients similar treatment to trained therapists.

However, the limited functionality of these robots and their high cost have reduced their availability, and the field is still in its infancy. To advance the field, this paper presents research into the actuators used in rehabilitation robots and potential control systems which may improve their functionality.

The type of actuator used in a rehabilitation robot is important for several reasons. They must be capable of repeated, accurate motion to provide the treatment necessary for patients requiring rehabilitation and be able to apply sufficient torque to the patient to move their limb along the required motion, or to resist their motion in the case of active resist exercises but be controllable such that the torque output does not exceed safe values. Specifics of actuator design and control for rehabilitation robotics are detailed in [3]. Another desirable factor is intrinsic compliance, which can help increase safety and comfort, allow users a degree of freedom of motion, as well as increase the transparency of the system by reducing patient–machine interaction forces. Several compliant actuators exist which are popular in the field of rehabilitation robotics, including Series Elastic Actuators (SEAs) and Pneumatic Artificial Muscles (PAMs), but controlling these actuators comes with its own problems. Due to the nonlinear nature of intrinsically compliant actuators, they act dynamically to input rather than linearly. This makes more traditional controllers like PID less effective in providing accurate motion. Reviews of soft and compliant actuators in rehabilitation robotics are presented in [4,5].

The use of pneumatic actuators in lower-limb rehabilitation robots has grown over the past few years, as explored in the review presented in [6]. Various pneumatic actuators are popular in the field for their compliant dynamics and interactive nature, including McKibben artificial muscles, rubbertuators, air muscles, pneumatic artificial muscles, and pneumatic muscle actuators. It is also mentioned in this review that while PID controllers are suitable for controlling pneumatic actuators, the nonlinear dynamics and complexity of the actuators may not be accurately controlled without further integrated control paradigms. Among those mentioned are various adaptive controllers using PID, a neuro-fuzzy network controller, neural-network based control and sliding mode control. One final suggestion states that a more complicated controller may not be the best approach to this problem, as additional sensors and impedances would increase the overall complexity of the system and make it more difficult to use, and a simpler controller is preferred.

This sort of compliant actuator is used for various purposes in the field, and in different configurations. The robot presented in [7] uses an antagonistic pair of pneumatic muscles to control a finger rehabilitation robot. This system also uses a magnetorheological dampener, and in experiments both open- and closed-loop PID control systems are implemented. A powered ankle–foot orthosis (PAFO) using a pair of PAMs to provide two degrees of freedom is presented in [8]. This system uses a sliding-mode controller for actuator control. The system presented in [9] consists of three separate pneumatic subsystems for body weight support, postural support, and gait orthosis. This system is a full-body gait rehabilitation system and uses an Interval Type-2 Fuzzy Sliding Pulse-Width Modulation Control to allow for proper control of the nonlinear pneumatic systems. A control system is developed in [10] which incorporates the RBF neural network and sliding mode control to ensure adaptive and disturbance-resistant control of a single-degree-of-freedom rehabilitation robot using a pair of antagonistic PAMs. The robot is designed to function for multiple different limbs. A neural network-based controller is also used in [11] for a hand rehabilitation robot utilising flexible pneumatic muscles. The intelligent controller has adaptive elements allowing it to accurately control the position of the orthosis despite interference forces and the nonlinearity of the actuators. Another control scheme designed to accurately control pneumatics is presented in [12] with a multiple-input single-output model free-adaptive control scheme. This controller is applied to a single DOF multi-joint rehabilitation robot

which uses two PAMs similar to [8]. Experiments presented in [13] aim to determine the dynamic characteristics of PAMs with the intention of accurate servo control. Nonlinear pressure flow values are compensated for using a lookup table, and position control is achieved using a model-based PID controller. This simple control structure achieved good closed-loop regulation and position tracking.

As shown in the literature, there is sufficient interest in pneumatic actuators in the field of rehabilitation robotics to make it an area of growing research, particularly the problem of accurate control. Accurate, reliable, and flexible motion tracking in a rehabilitation robot is essential for its effective use in a real rehabilitation setting. As professional therapists set the desired exercise motions, it is important to have them be properly followed to ensure proper rehabilitation outcomes for patients, as well as ensure their safety in using a robot. A control system able to perform well under many different input requirements also makes the robot more flexible in the conditions and stages of recovery it can treat. However, while good control performance is shown in many studies, there is a lack in comparison data between different intelligent control approaches in PAM-actuated rehabilitation robotics.

To address this limitation and investigate a novel application of prediction and optimisation algorithms, this paper presents preliminary implementation and testing of a Model Predictive Control (MPC) scheme utilising a Particle Swarm Optimisation-based (PSO) modelling method to generate dynamic models of the actuator, tested experimentally on a single-PAM setup in a simple hanging position as a feasibility study for eventual application of the controller to a PAM-driven rehabilitation robot. The development and experimental validation of the PSO-based modelling method is presented in [14]. This approach involves a phenomenological approximation of the PAM to a mass-spring-damper system, optimising the three parameters using a PSO algorithm in a three-dimensional search space. Based on preliminary testing, the linear model generation method, in which a single model is used to approximate the PAM's motion over a specific pressure input range, will be used here. The switching issue present in the piecewise and inflate–deflate modes has a magnified effect when used in conjunction with a control algorithm; as such, the linear method was considered the best approach for motion accuracy.

In order that this controller is properly validated in comparison with other controllers commonplace in the field, two other intelligent control strategies were applied to the system, as well as one classical control strategy: iterative learning control (ILC), Particle Swarm Optimised Proportional Integral Derivative Control (PSO-PID), and classical PID control. While PID control is generally regarded as unsuitable for use with nonlinear systems based on its linear nature and poor approximation of time-variant systems, its use is prevalent in control theory as a computationally and mathematically efficient algorithm. As such it will be included in this study as a comparison point to demonstrate the improved effectiveness of intelligent control on the PAM system.

MPC uses a dynamic model of the system to predict over a finite period the behaviour of the system and calculates the control output required to best optimise some cost function over that period. Using this predictive method, the time-variant characteristics of the system are accounted for, and nonlinearities can be included in the model of the system. This makes MPC a suitable controller for PAMs. However, the need for a model of the system makes it difficult to apply to complex systems and actuators. This is especially true for systems including uncertainties and multiple nonlinear factors such as PAMs, as models for such systems tend to include multiple assumptions and simplifications, giving model-based controllers an inherent trade-off between accuracy and computational efficiency. In [15], a switching model predictive controller is developed for a PAM for the purpose of testing a novel modelling method using PieceWise Affine model approximation. The PAM is initially estimated as a spring-damper system with a second-order differential equation

of motion, but in the piecewise modelling estimation the second-order element is ignored. A single PAM is set up vertically to measure displacement with no load attached. The controller, in comparison with the PID control of the same system, is found to have lower motion error values and faster convergence. A 2 DoF robot actuated by two antagonistic pairs of PAMs as presented in [16] which uses MPC for motion control. Simulations show that the controller provides accurate motion control with small errors and stable steady-state behaviour, with the model used for the system dynamics being a nonlinear eighth-order set of differential equations, with polynomial functions serving as the characteristics of the PAMs. A discrete-valued model-predictive controller dubbed DVMPC is presented in [17], and improved upon in [18], for motion control of a novel hybrid-pneumatic electric actuator and a double-acting pneumatic cylinder, respectively. Results from both studies show that novel controllers outperform the state-of-the-art sliding mode control by a significant margin. A lower limb orthosis powered by PAMs and controlled by a neural network-based nonlinear MPC is presented in [19]. In online experiments, the system meets requirements for each different participant, and the major concerns for inhibited system performance are the PAM's intrinsically slow rate of motion owing to the maximum allowed air flow into and out of it, and human-machine interaction sensing. MPC has been proven suitable for accurate motion control of PAMs; however, the modelling required for this control method produces another level of complexity, as this in itself is a separate area of study.

ILC is popular for use with nonlinear actuators as it does not rely on a complex model of the system, rather measuring the desired and actual outputs and iteratively calculating the best control signal based on the difference. This makes it suitable for time-varying systems, and there are many examples of its use in rehabilitation robotics using PAMs. The system RUPERT presented in [20] uses ILC for assistive reach-to-grasp tasks actuated by a series of PAMs, where its use is attributed to the need for a suitable controller for a nonlinear and time-varying system, as well as the repetitive nature of attempting the same task multiple times. Another rehabilitation/assistive robot utilising PAMs is presented in [21], in which uses an improved PID controller has issue with phase lag relieved using ILC to iteratively adjust control parameters, fixing this lag issue and reducing output error. A simulation of a PAM presented in [22], utilising ILC to account for the nonlinear and time-varying nature of the actuator, shows results proving the algorithm is capable of accounting for these characteristics, as well as uncertainties in the system. Based on the widespread use and research into ILC in the field of rehabilitation robotics and specifically its usefulness in controlling PAMs, it was chosen as a comparison for the developed MPC in this study.

Similarly to learning algorithms, optimisation is often used to improve the performance of controllers for complex systems, with PSO being a popular optimisation algorithm. PID is often the target of such optimisation as its functionality is heavily tied to how well the small number of parameters are tuned to the system it is to control. An upper limb exoskeleton robot using this method of optimised control is presented in [23] alongside a method using an improved version of classical PSO, Artificial Bee Colony Optimisation. A lower limb rehabilitation robot presented in [24] also uses improved PSO-PID control. Both these studies found their optimised controllers to be robust and have more accurate motion control. The study presented in [25] develops a dynamic neural network and PID controller for control of PAMs, the parameters of which are optimised using the PSO algorithm with Integral Time Absolute Error as the objective function. Another study using a similar technique is presented in [26] with adaptive and classical synergetic controllers developed for a robotic arm actuated by a PAM.

The experiments presented in this paper aim to test and compare the developed MPC with classical ILC, PID, and PSO-PID in controlling the actuation of a single PAM in online control experiments, for future implementation to a complete PAM-drive rehabilitation robot. The main results for determining effectiveness will be motion tracking accuracy, response to disturbance, and smoothness of motion, as these are necessary for the controller to function well as part of a rehabilitation robot utilising PAMs for actuation. The experiments will take place on a single PAM set up to accurately measure displacement. Weights will be used to apply resistive force to the actuator, as well as to simulate disturbances to the system. Each controller will be implemented and tested with the same test scheme, and the displacement error values will be measured and analysed to determine how well they perform comparatively.

The contributions of this study include development and implementation of a model predictive controller for accurate PAM motion using the PSO-based modelling method presented in [14] to generate dynamic models of the system, experimental data for comparison of the performance of the MPC as well as other popular controllers in the field of rehabilitation robotics—ILC, PID, and PSO-PID—using an experiment plan designed to simulate the motion and load requirements of PAMs in a rehabilitation robot, as well as a comparative discussion comparing the relative strengths and weaknesses of each controller. Based on the limited examples of comparative studies, this experiment including multiple commonplace controllers will help in quantifiably demonstrating the relative benefits and shortcomings of these controllers and help fill this gap in the literature. The overall aim of this research is to determine the feasibility of the developed MPC, as well as classical ILC and optimised PID controller, for accurate motion control of PAMs in a rehabilitation robot setting, with the future aim to implement the developed algorithms in a PAM-driven rehabilitation robot.

2. Materials and Methods

2.1. Controllers

2.1.1. Model Predictive Control (MPC)

MPC uses a model of the system as well as prior knowledge of the input sequence to predict the movement of the actuator in advance for a given future time period. This allows for accurate control of nonlinear systems for any input sequence and is therefore usable for various applications. MPC also has the option of constraining many aspects of the control sequence, including minimum and maximum control values for both the control output and the predicted control output, as well as minimum and maximum values for the rate of change of control values. However, the necessity of a mathematical model of the system can cause issues in development, especially for a complex system such as a PAM, as these models often include simplifications and assumptions resulting in inaccuracies. The controller also requires that the entire input sequence be known beforehand, and the predictive nature prevents it from being used in modes other than passive movement, such as resistive training in rehabilitation. The structure of the MPC algorithm is shown in Figure 1.

At each sampling period t the state of the system is taken and via an optimization function, a cost-minimising control output is calculated for a time horizon defined by the prediction period T . Once a cost-minimal control trajectory starting at the sampled state and for the time period $[t, (t + T)]$ is found, the first step of this strategy is applied to the system. The remainder of the calculated control trajectory is discarded, and the process is repeated from the new system state. While MPC is an optimal control strategy as it uses a cost optimisation method for control, the process by which it calculates this control is not optimal as a great many calculations are discarded.

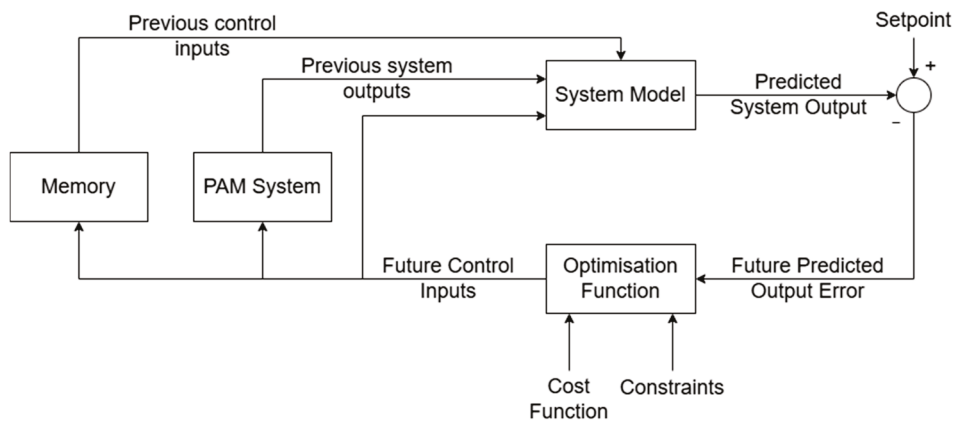


Figure 1. MPC structure. The system model is generated according to the methods described in [14].

The optimisation algorithm used here is linear quadratic programming, the cost function of which is described by Equation (1):

$$J = \sum_t^T q \left(\hat{y} - s \right)^2 + \sum_t^T r \Delta u^2 \quad (1)$$

where t is the current time step, T is the prediction period, q is the output error weight parameter, $\hat{y}$ is the predicted system output, s is the setpoint, r is the control weight parameter, and Δu is the predicted change in the control value such that $\Delta u = u_k - u_{k-1}$ with u_k being the control value taken at time step k . For this MPC implementation, the output error weight was set to 1 and the control weight was set to 20.

Based on this cost function, the optimal control is achieved by solving Equation (2) for u :

$$\frac{\delta J}{\delta u} = 0 \quad (2)$$

As MPC relies heavily on the accuracy of the model of the system to properly optimise potential control solutions, it is important the model used approximates the PAM as closely as possible. As discussed in [14], modelling PAMs is particularly challenging due to their dynamic behaviour. The PSO-based modelling method used there will be employed here to generate mathematical models for the MPC based on the proposed mass-spring-damper approximation. To get a more accurate model response without the undesirable jumping from a piecewise model, a single linear phenomenological model will be generated for each required motion, accounting for the position- and time-variant behaviour of the PAM over different stroke lengths and pressure input ranges.

2.1.2. Iterative Learning Control (ILC)

The ILC improves tracking performance over multiple iterations of the same input sequence, thereby progressively reducing errors. Using this control algorithm, long-term stability and performance is improved; however, the initial learning period is generally unstable and inaccurate. Therefore, reducing this initial period by speeding up the learning rate can improve the functionality of the controller. This control method is model-free, as the controller output is calculated using error feedback. This is a desirable trait for controllers of nonlinear systems as it removes the need for a complex mathematical model, which would likely include simplifications and assumptions.

The standard equation for control output for ILC is shown in Equation (3):

$$u(t) = u_{old}(t) + pe_{old}(t) \quad (3)$$

where $u(t)$ is the new control signal, $u_{old}(t)$ is the control signal at the same time step within the previous learning period, $e_{old}(t)$ is the error at the same time step within the previous learning period, and p is the learning rate parameter which is manually tuned.

2.1.3. Particle Swarm Optimised PID Control (PSO-PID)

PID control is generally regarded as unsuitable for nonlinear systems such as PAMs as it treats the input–output relationship of the system as position- and time-invariant. Introducing PSO algorithms to optimise the parameters of the PID controller can both improve this shortcoming by calculating optimal parameters based on the behaviour of the PAM over the required motion. The optimisation algorithm is run offline to generate parameters for the PID controller for online experiments.

The equation for velocity update of each particle for classical PSO is shown in Equation (4):

$$V_i(k+1) = WV_i(k) + C_1 \text{Rand}(X_{i\text{best}}(k) - X_i(k)) + C_2 \text{Rand}(G_{\text{best}}(k) - X_i(k)) \quad (4)$$

The equation for position update is shown in Equation (5):

$$X_i(k+1) = X_i(k) + V_i(k+1) \quad (5)$$

where i is the current particle; k is the current iteration of the algorithm; V is the particle's velocity in the search space; X is a particle's position in the search space quantified by its parameters; Rand is a random value between 0 and 1; $X_{i\text{best}}(k)$ is the position of the current particle which achieved the best (lowest) fitness; $G_{\text{best}}(k)$ is the global best position of all particles in the swarm; and W , C_1 , and C_2 are algorithm parameters that are tuned manually.

To use PSO to optimise parameters for PID control, a 3-dimensional search space is used, optimising the parameters K_c , T_i , and T_d . To converge on an optimal solution, the objective function used for swarm fitness is the RMSE of motion tracking over a single input wavelength.

2.2. Experimental Setup

The system designed for testing the developed controllers includes a single PAM setup in a vertical position, with the fixed end connected to a frame and the unaffixed end connected to a linear encoder as the output measure of these experiments is the actuator's displacement. Pressure input and output to the PAM is controlled by a proportional pressure regulator, the input voltage of which is the controlled variable of the system. Figure 2 shows a picture of the PAM setup with the encoder sensor and external weight attachment.

The actuator to be used in these experiments is a FESTO DMSP-20-400N PAM. The model of the linear encoder is a FESTO MLO-POT-300-TLF with a resolution of 0.01 mm and stroke length of 300 mm, both of which are sufficient for the system. The proportional pressure regulator used for controlling pressure input to the PAM is a FESTO VPPM-6L-L-1-G18-0L6H. FESTO is based in Esslingen am Neckar, Germany. The system is held in place with a frame consisting of slotted aluminium extrusion and the PAM is positioned vertically to ensure motion and forces are not adversely affected by the actuator's intrinsic compliance regarding gravity.

To control the system, a National Instruments RoboRIO is connected to a desktop PC and coded using National Instruments LabVIEW 2016. National Instruments is headquartered in Austin, Texas, United States. The output of the displacement encoder is input to the RoboRIO to be measured on the display.

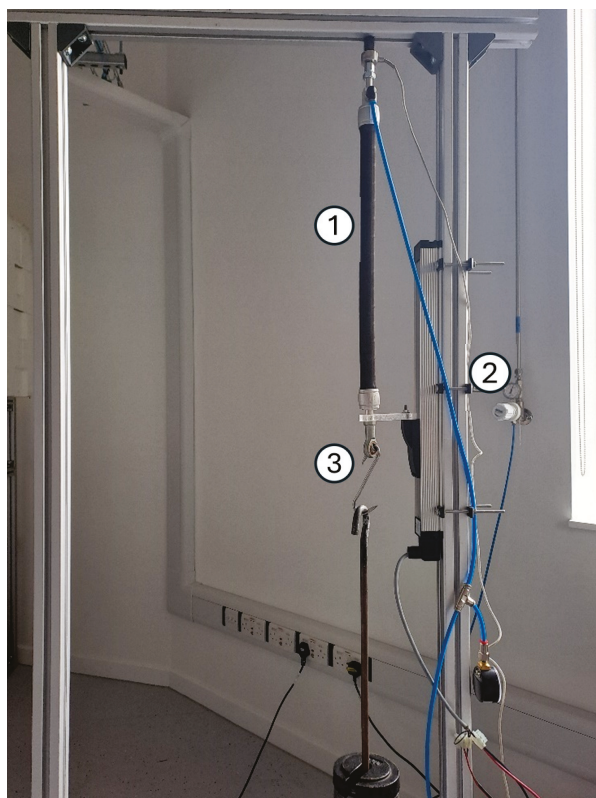


Figure 2. The experimental setup, showing the FESTO DMSP-20-400N PAM (1), FESTO MLO-POT-300-TLF displacement encoder (2), and external load attachment (3).

Figure 3 shows a diagram of the experimental setup, including communication with the embedded controller and computer.

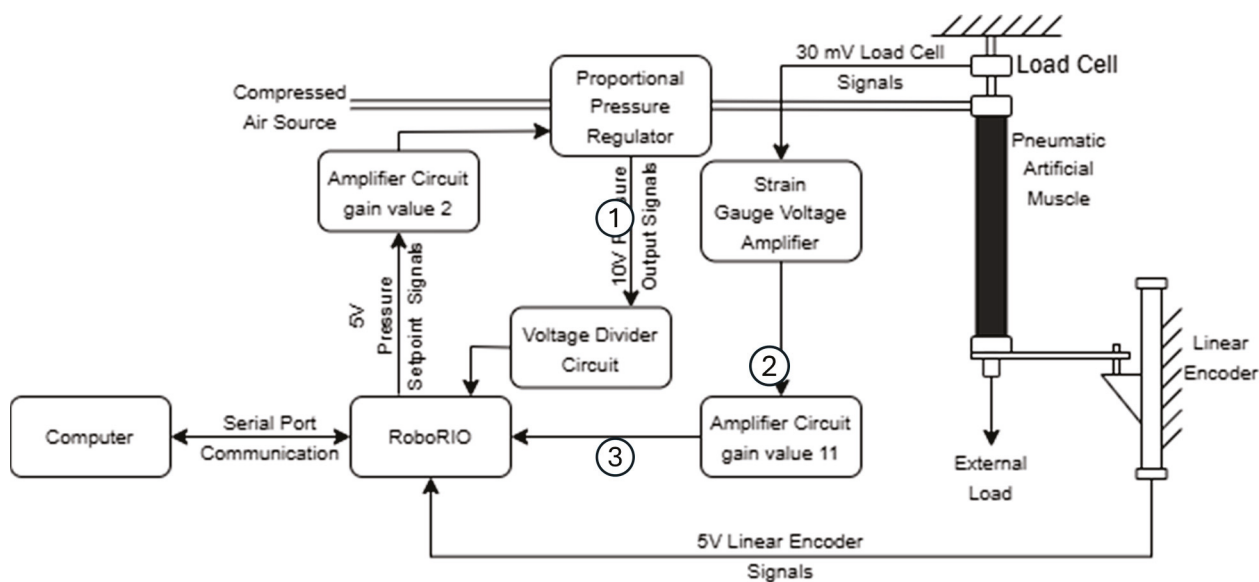


Figure 3. A diagram of the experimental setup, circuitry, and computer communications.

2.3. Experiment Plan

Each experiment will involve a specific motion as input to each controller, along with the addition of external weights applied to the PAM, and adding/removing them at certain points during motion. Table 1 shows the experiment outline as well as the main purpose of

each set of experiments. Each experiment, including input motion setpoints and weights used, will be performed unaltered for each controller to ensure the results are comparable.

Table 1. Experiment Outline.

Experiment	Experiment Purpose	Motion and Weights
Continuous Motion	Determine controller's motion accuracy with no disturbance for different types of motion	Sin wave, 0 cm–10 cm Sin wave, 0 cm–5 cm Sin wave, 5 cm–10 cm Sin wave, 0 cm–10 cm, rapid motion Triangle wave, 0 cm–10 cm Square wave, 0 cm–10 cm
Continuous Motion with Weights	Determine the effect of added weight on controller's motion accuracy	Sin wave, 0 cm–10 cm, 5 kg load Sin wave, 0 cm–10 cm, 10 kg load Square wave, 0 cm–10 cm, 5 kg load Square wave, 0 cm–10 cm, 10 kg load Sin wave, 0 cm–10 cm, rapid motion, 10 kg load Sin wave, 0 cm–5 cm, 10 kg load Sin wave, 5 cm–10 cm, 10 kg load
Continuous Changing Motion	Determine the controller's response to a change in continuous motion, how quickly they can adapt Changes made after 6 wavelengths	Sin wave, 0 cm–4 cm changed to 0 cm–8 cm Sin wave, 0 cm–10 cm changed to 2.5 cm–7.5 cm Sin wave, 0 cm–5 cm changed to 5 cm–10 cm Sin wave, 0 cm–10 cm, slow motion changed to rapid motion Sin wave, 0 cm–10 cm, rapid motion changed to slow motion
Static with Disturbance	Determine controller's response to disturbance with no noise or motion Performed with static 5 cm setpoint, weights added/removed at 5 s	5 kg load added 10 kg load added 5 kg load removed 10 kg load removed
Continuous Motion with Disturbance	Determine controller's response to disturbance during motion	Sin wave, 0 cm–10 cm, 10 kg load added after 4 waveforms Sin wave, 0 cm–10 cm, 10 kg load removed after 4 waveforms Sin wave, 0 cm–10 cm, 5 kg load added and removed after each waveform

2.4. Algorithm Parameters

2.4.1. MPC Model Generation

The PSO-based modelling method presented in [14] is used here to generate dynamic models of the PAM for use in the MPC algorithm. A PSO algorithm with a swarm size of 50, $W = 0.9$, $C_1 = C_2 = 2$, and run for 20 iterations was used to generate each model using the method described in [14]. The fitness value was calculated using the actual PAM motion with a sin wave voltage input to the pressure regulator, the amplitude of which was based on the steady-state displacement of the PAM at certain voltage inputs, as shown in Figure 4, such that the required displacement upper and lower bounds for the comparative experiment were reached during model generation. Using this method, a set of model parameters were generated which best described the PAM's behaviour for each required motion in the experiment plan, allowing for more accurate control through the MPC algorithm. The parameters of these models are shown in the MPC model parameters column in Table 2.

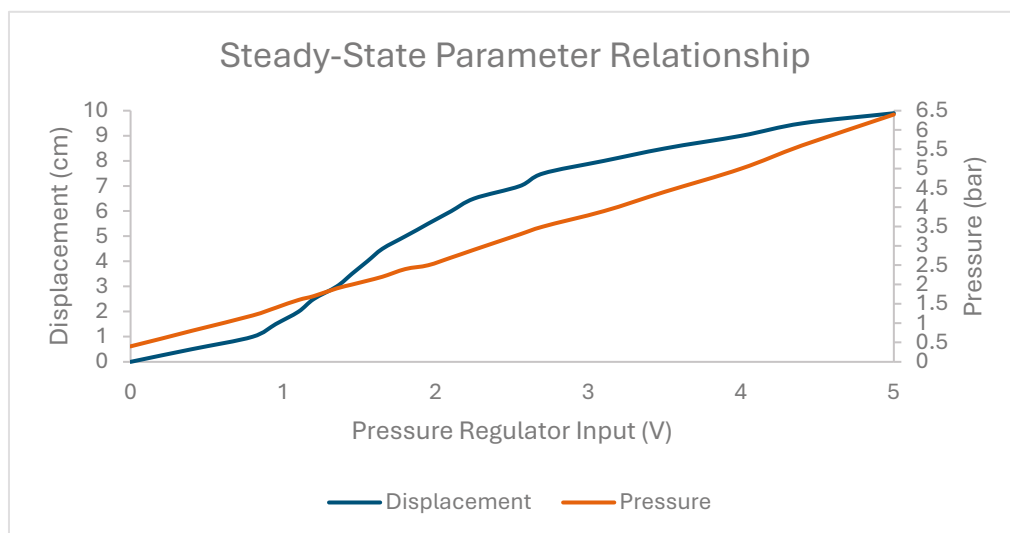


Figure 4. Steady-state relationships between voltage input to the pressure regulator, internal air pressure in the PAM, and actuator displacement.

Table 2. PID control parameters and MPC model parameters.

Waveform	Amplitude (cm)	Offset (cm)	Frequency (Hz)	PSO-PID Parameters			MPC Model Parameters		
				K_c	T_i	T_d	P_1	P_2	P_3
Sinusoidal	10	0	0.25	0	0.801752	0.0191877	8.18×10^{-6}	0.182535	0.534393
Sinusoidal	5	0	0.25	0	0.34711	0.00266562	0	0.460018	0.191448
Sinusoidal	5	5	0.25	0	1.0466	0.0187886	3.45×10^{-6}	0.741741	0.012203
Sinusoidal	10	0	1	0	0.801752	0.0191877	8.18×10^{-6}	0.182535	0.534393
Triangular	10	0	0.25	0	0.570082	0.00962227	9.15×10^{-6}	0.182535	0.534393
Square	10	0	0.25	0	0.885228	0.00971446	4.96×10^{-6}	0.182535	0.534393
Sinusoidal	10	0	0.25	5	0.801752	0.0191877	8.18×10^{-6}	0.182535	0.534393
Sinusoidal	10	0	0.25	10	0.801752	0.0191877	8.18×10^{-6}	0.182535	0.534393
Square	10	0	0.25	5	0.885228	0.00971446	4.96×10^{-6}	0.182535	0.534393
Square	10	0	0.25	10	0.885228	0.00971446	4.96×10^{-6}	0.182535	0.534393
Sinusoidal	10	0	1	10	0.801752	0.0191877	8.18×10^{-6}	0.182535	0.534393
Sinusoidal	5	0	0.25	10	0.34711	0.00266562	0	0.460018	0.191448
Sinusoidal	5	5	0.25	10	1.0466	0.0187886	3.45×10^{-6}	0.741741	0.012203
Sinusoidal	4	0	0.25	0	0.442617	0.00526149	5.98×10^{-6}	0.101338	0.626386
	8	0	0.25	0	0.756974	0.00641648	5.38×10^{-6}	0.503874	0.154515
Sinusoidal	10	0	0.25	0	0.801752	0.0191877	8.18×10^{-6}	0.182535	0.534393
	5	2.5	0.25	0	0.626517	0.00597624	7.00×10^{-6}	−0.10410	0.890396
Sinusoidal	5	0	0.25	0	0.34711	0.00266562	0	0.460018	0.191448
	5	5	0.25	0	1.0466	0.0187886	3.45×10^{-6}	0.741741	0.012203
Sinusoidal	10	0	0.25	0	0.801752	0.0191877	8.18×10^{-6}	0.182535	0.534393
	10	0	1	0	0.801752	0.0191877	8.18×10^{-6}	0.182535	0.534393
Sinusoidal	10	0	1	0	0.801752	0.0191877	8.18×10^{-6}	0.182535	0.534393
	10	0	0.25	0	0.801752	0.0191877	8.18×10^{-6}	0.182535	0.534393

Table 2. Cont.

Waveform	Amplitude (cm)	Offset (cm)	Frequency (Hz)	PSO-PID Parameters			MPC Model Parameters		
				K_c	T_i	T_d	P_1	P_2	P_3
Constant	0	5	0	5	0.632498	0.0134572	1.10×10^{-5}	−0.10410	0.890396
Constant	0	5	0	10	0.632498	0.0134572	1.10×10^{-5}	−0.10410	0.890396
Constant	0	5	0	5	0.632498	0.0134572	1.10×10^{-5}	−0.10410	0.890396
Constant	0	5	0	10	0.632498	0.0134572	1.10×10^{-5}	−0.10410	0.890396
Sinusoidal	5	2.5	0.25	10	0.626517	0.00597624	7.00×10^{-6}	−0.10410	0.890396
Sinusoidal	5	2.5	0.25	10	0.626517	0.00597624	7.00×10^{-6}	−0.10410	0.890396
Sinusoidal	5	2.5	0.25	5	0.626517	0.00597624	7.00×10^{-6}	−0.10410	0.890396

2.4.2. PID Parameters

The parameters used for the classical PID controller were $K_c = 0.6$, $T_i = 0.01$, and $T_d = 1e^{-5}$. These parameters were tuned manually.

2.4.3. ILC Parameters

The learning rate used for the ILC in each experiment was $p = 0.2$. This value was tuned manually to optimise rate of convergence to the setpoint while minimising overfitting.

2.4.4. PSO-PID Parameters

To improve on the classical PID algorithm, PSO was used to generate optimal parameters for the three control values. Based on the dynamic behaviour of the PAM across its stroke length, as well as keeping the controller as computationally simple as possible while maintaining accurate motion tracking, a set of parameters, each of which included values K_c , T_i , and T_d , was generated for each motion required by the comparative experiment plan. This allowed for the most optimal PID control available based on the algorithm for each experiment.

Each set of parameters was generated using a PSO algorithm with a swarm size of 10, $W = 0.9$, $C_1 = C_2 = 2$, and run for 10 iterations. The position values of each particle corresponded to the K_c , T_i , and T_d parameters of the PID controller. The fitness of each particle was calculated as the RMSE between the desired setpoint motion of the PAM displacement and the actual PAM displacement under PID control, using that particle's parameter values, over two wavelengths of motion. The resulting parameter sets are shown in the PSO-PID parameters column in Table 2.

3. Results

The displacement of the PAM under each control scheme was measured for each experiment and overlaid for comparison, as shown in Figure 5. The displacement error for each experiment was also calculated and overlaid for each experiment. Figures 6–12 show this error results from some of the experiments best demonstrating the relative performance of the controllers.

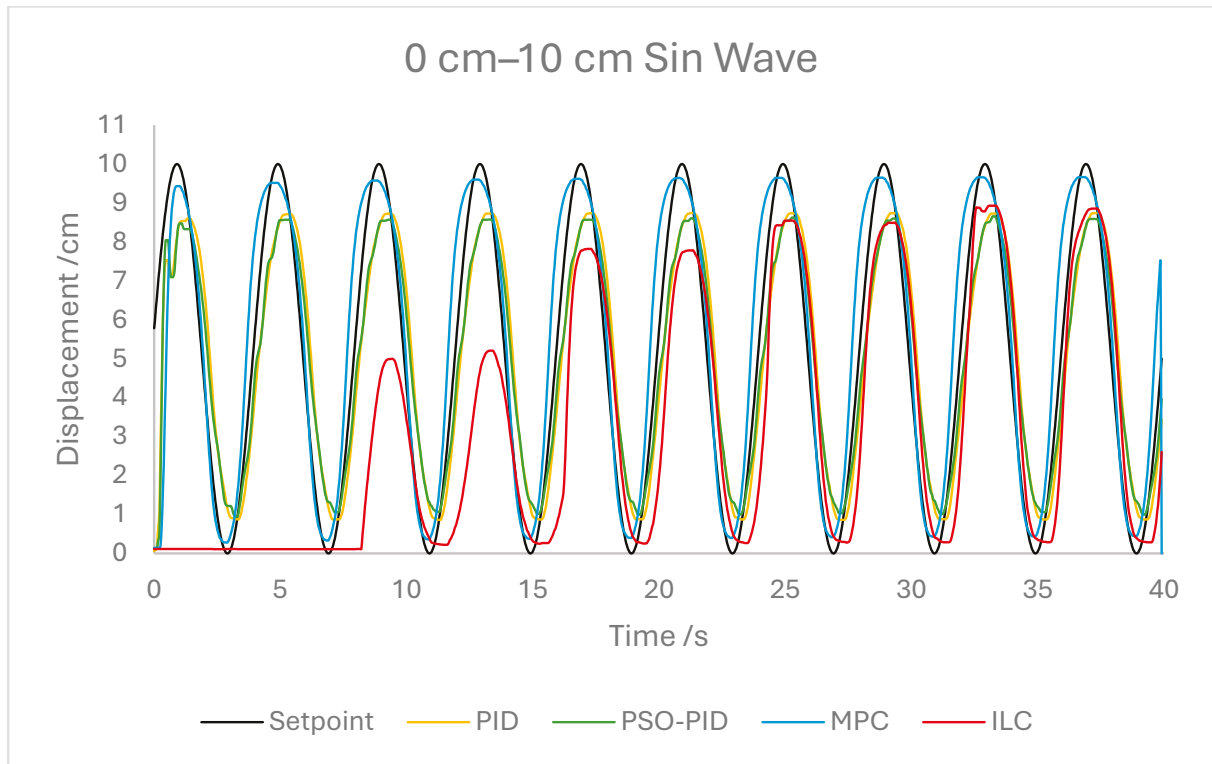


Figure 5. Graph showing the setpoint waveform and PAM displacement under each control scheme during the 0 cm–10 cm sin wave experiment.

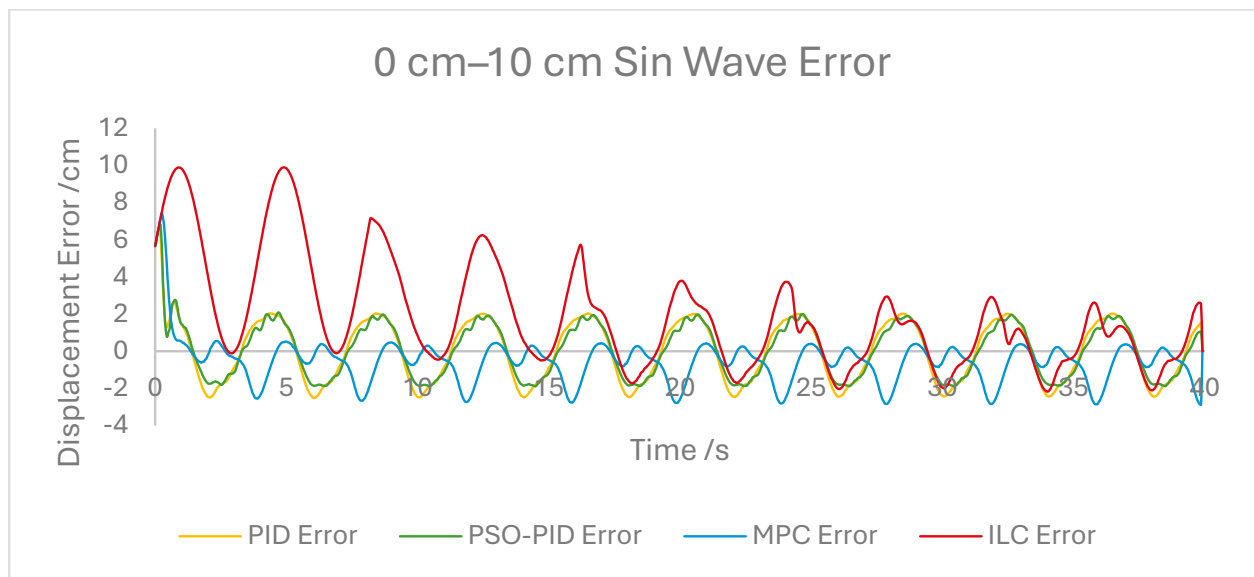


Figure 6. Graph showing displacement error of the PAM under each control scheme during the 0 cm–10 cm sin wave experiment.

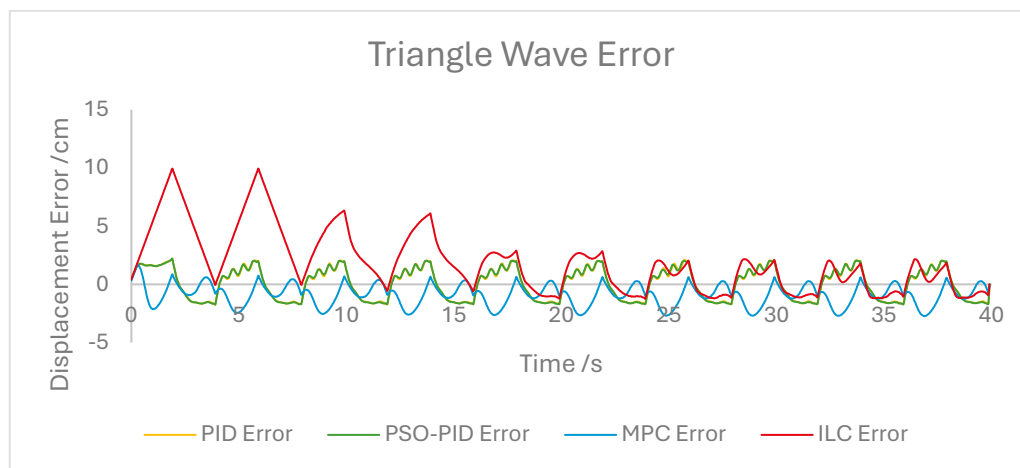


Figure 7. Graph showing displacement error of the PAM under each control scheme during the triangle wave experiment.

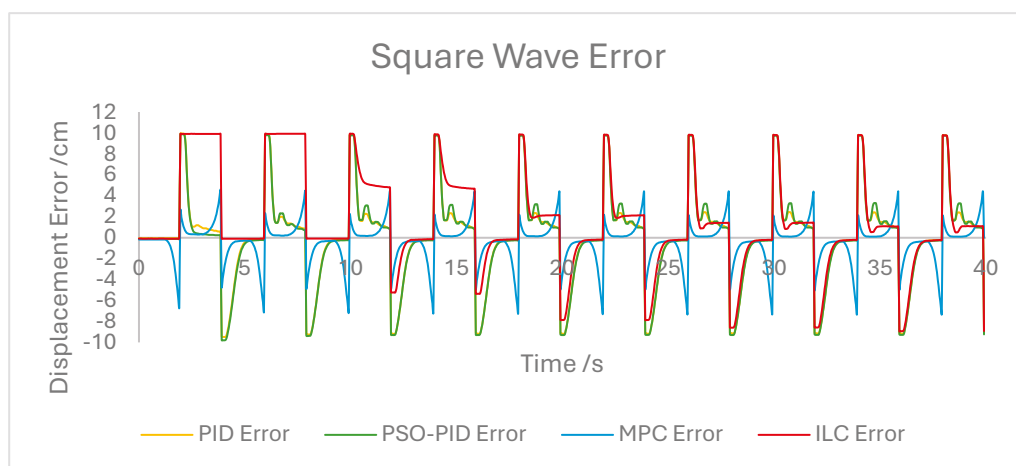


Figure 8. Graph showing displacement error of the PAM under each control scheme during the square wave experiment.

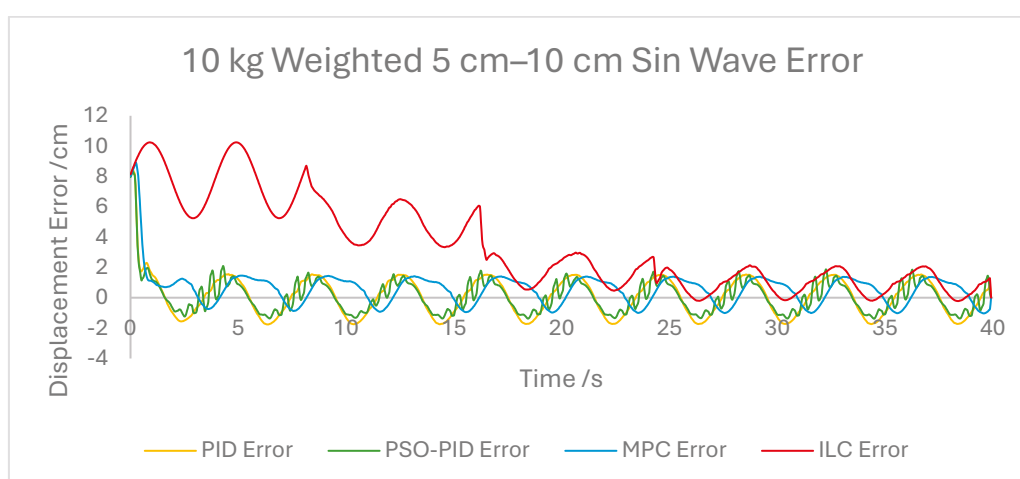


Figure 9. Graph showing displacement error of the PAM under each control scheme during the 10 kg-weighted 5 cm–10 cm sin wave experiment.

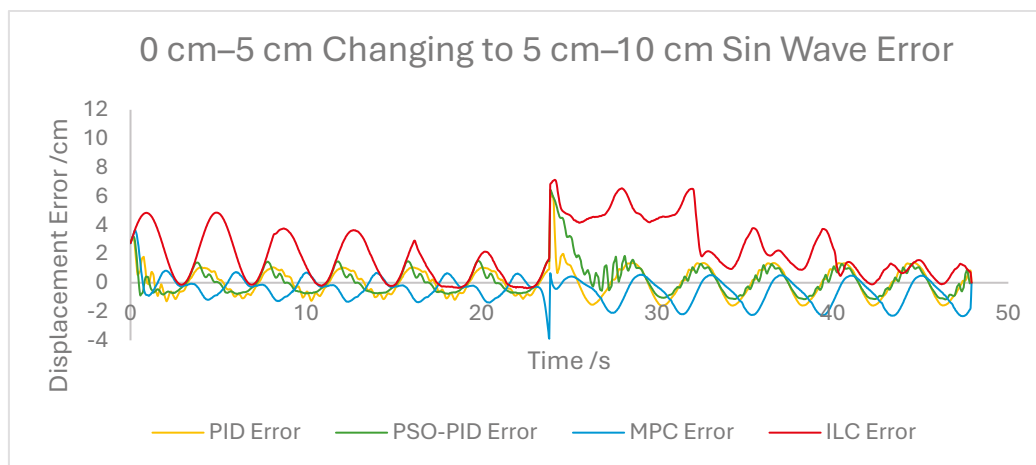


Figure 10. Graph showing displacement error of the PAM under each control scheme during the 0 cm–5 cm changing to 5 cm–10 cm sin wave experiment.

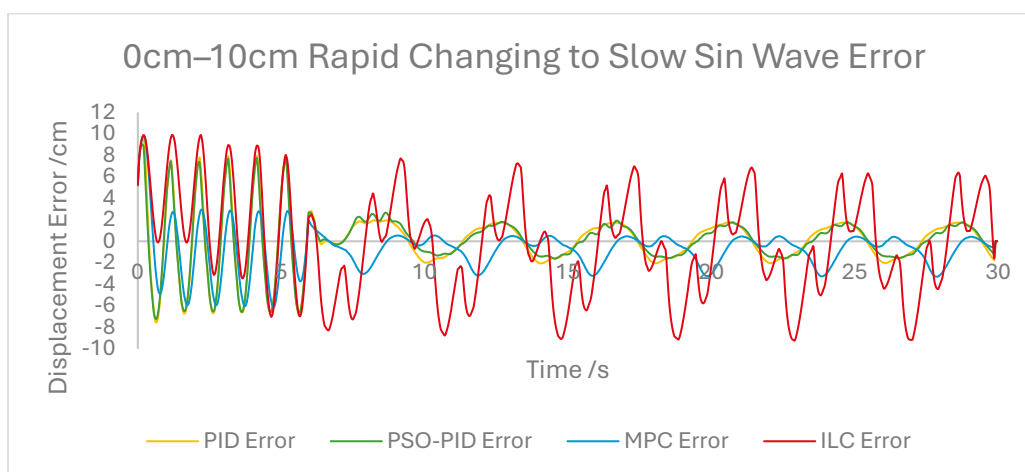


Figure 11. Graph showing displacement error of the PAM under each control scheme during the rapid changing to slow motion 0 cm–10 cm sin wave experiment.

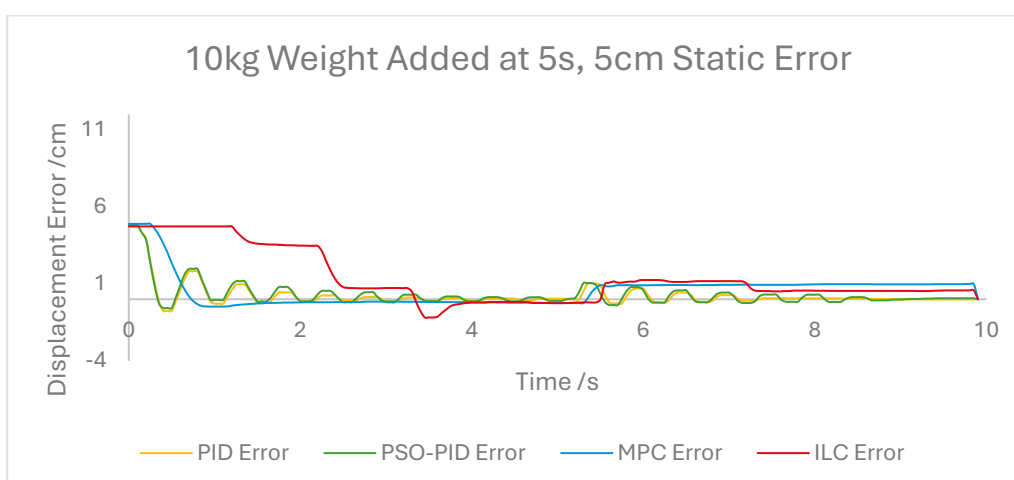


Figure 12. Graph showing displacement error of the PAM under each control scheme during the 10 kg added weight experiment.

4. Discussion

The dynamic properties of the PAM heavily impact the accuracy of motion under all control schemes at a high-velocity input, such as during the rapid sin wave experiments,

in which a 1 Hz setpoint waveform is used rather than the 0.25 Hz used in the other experiments, as well as the square wave experiments. Rather than a fault in the controllers, this is mostly a reflection of the maximum change in displacement the actuator is capable of achieving. This also has an effect under a triangle wave setpoint, as constant velocity is difficult to achieve with dynamic input/output behaviour. The higher frequency experiment, while not an accurate representation of actuator requirements in a rehabilitation setting, was performed to test the higher limits of speed response for the PAM and controllers. The error values for these experiments were generally the highest; however, the comparison between the controller is useful as will be discussed.

The performance of the PID and PSO-PID controllers were generally consistent with each other. Both exhibited oscillating behaviour during most motions, including during constant displacement experiments and during the stationary points of the square wave setpoints. This was exacerbated with the addition of external loads, especially noticeable in the static experiments where the oscillation was mostly present before the weights were removed and after they were added. Both PID control schemes had a poor response time under square wave setpoints compared to the other controllers, with noticeably larger errors at the changing points in the waveform as well as slower settling time; however, the steady-state error at the static points was generally small. The poor response time can also be seen in the sin wave experiments with the PAM motion lagging behind the setpoint. The PSO-PID controller showed lower error values in all experiments than classical PID, and the oscillating behaviour was less pronounced, except during the upper stroke length of the PAM and during the triangle wave setpoint. This behaviour is likely due to a closer fitting to the setpoint at points where the nonlinear nature of the PAM is most pronounced, causing the controller to quickly switch between increasing and decreasing pressure input in an attempt to meet the desired displacement.

The behaviour of the ILC was as expected in all experiments in terms of learning, with convergence to a steady state reached after eight wavelengths, or four iterations of the learning algorithm. Once a steady state is reached the error is typically similar in magnitude to that of the PSO-PID controller, but with no evidence of oscillation about the setpoint. Motion under the ILC is generally smoother than under PID control. ILC suffers from similar response-time issues to PID control, with the PAM motion lagging behind the setpoint even after a steady state is reached. The response time during square wave motion is slightly better than that of PID, with lower settling time and notably no oscillation at the static points, but instantaneous errors are similar in magnitude at the changing points of the waveform. Under external load the ILC performs well, with little impact on the learning rate or the overall motion accuracy. Addition and removal of weights during motion and during static displacement is accounted for by the learning algorithm; however, with a constant setpoint the steady state error under ILC is the worst of the controllers tested here. Under changing setpoints the ILC can respond to the change in a similar time to the initial learning period, but this does result in worse overall error. The learning algorithm does not respond properly to changes in the frequency of the setpoint during motion, with the initial frequency maintained throughout without changing to match the setpoint. This is due to the learning period being based on the wavelength of the setpoint. A more sophisticated learning algorithm could improve on this with a better-defined learning period.

Overall, based on average error values across the experiments' runtimes as well as maximum error magnitudes, the motion of the PAM under MPC is the most accurate. The response time issue present in the other controllers tested here is removed by the predictive algorithm, although in some cases the actual motion precedes the setpoint, which is a unique issue. With square wave input this is most noticeable, as the PAM motion under MPC changes direction before the changing points of the waveform, resulting in overall

lower error values. Under most setpoints the error is lowest under MPC with smoother motion than PID and, as to be expected, a faster settling time than ILC. One major downside of this implementation of MPC is that the model used does not consider external loads to the system, and as such the controller does not change input based on additional loads to the PAM, resulting in larger error values when weights are added. Under rapid motion the predictive algorithm works somewhat against the PAM motion. The system itself is incapable of changing velocity at the rate required by the setpoint, usually resulting in a lagging motion which attempts to reach the peak of the sin wave input. The MPC predicts rapid changes in the setpoint before they occur and changes the direction of motion in advance. While this does result in overall lower error values under MPC than other controllers, the maximum displacement of the PAM is much smaller in practice due to this interaction, which is usually undesirable when displacement accuracy is the priority. The predictive algorithm is capable of adjusting to changes in setpoint waveforms seamlessly. PAM motion immediately before the change in waveform is adjusted to best fit the requirements after the change, so error values are only minorly affected, there is little or no increase in error between the different setpoints, and motion after the change is just as accurate as before.

While the predictive and learning control systems used in this study show promise in accurate motion control for a single actuator, alternative control methodology is required in many rehabilitation robotics applications. Impedance control, such as that presented in [27,28], uses force sensor measurements to determine actuator motion. This feedback system is required in active rehabilitation, in which a user applies force to the robot and the motion is assisted or resisted by the system. This would be impractical for the controllers used here to achieve in their current configuration, so they are not sufficient for every control need of a rehabilitation robot.

5. Conclusions

This study presents an intelligent control scheme designed to overcome the nonlinearity of PAMs, Model Predictive Control using PSO-based dynamic modelling, which was implemented on a single PAM setup to test for validity. Three other controllers which are popular in the field of rehabilitation robotics, ILC, PID, and PSO-PID, were also investigated for quantifiable comparison of their performance.

An experiment scheme was designed to test the behaviour of each control scheme under conditions, as would be required in a rehabilitation setting. Displacement of the PAM was measured for each different experiment setpoint and external loading under each of the four control schemes, and the displacement error calculated and compared. Based on these comparisons, it was found that the PID controller performed worst in terms of error magnitude and response time, with undesirable oscillating behaviour in many cases, which was worsened by the addition of weights. Optimised parameters using PSO slightly improved the error values and response times but did not remove the oscillations. The ILC reached a steady state after learning for four iterations in each experiment, with smoother motion and smaller error values than PID. Performance was also not inhibited by external loading, and changes to the setpoint and external loading during motion were accounted for in the next learning iteration. Response time was still slow, however, and the algorithm was incapable of adjusting to changes in frequency. The MPC had the best performance, with smooth motion during all experiments and the overall lowest error values. The prediction algorithm allowed motion in advance of changing setpoints and maintained low error values after changes as well. Due to the method of model generation for the MPC and the lack of feedback, the algorithm was unable to adjust for external loads, and error values were negatively affected by weights.

The results gained in this study prove conclusively that machine intelligence can greatly improve the performance of control systems for PAMs in terms of tracking accuracy, response time, and motion smoothness. Both learning and predictive control methods show excellent performance in most scenarios. Despite being a standard implementation of learning control, the ILC algorithm used in this study showed rapid convergence to accurate motion as well as the ability to sufficiently reject external load and disturbance. The use of additional learning parameters and robust elements could further improve its performance. The predictive nature of MPC especially shows promise as it mitigates many issues which arise in controlling complex actuators like PAMs, including lagging behind the setpoint caused by a limited flow rate of air into the actuator, and the PAM's tendency to oscillate about setpoint values as it repeatedly attempts to over-adjust. These preliminary results suggest that this implementation of MPC is suitable for further study and future application to a PAM-driven rehabilitation robot. It could also be suitable for other robotics applications, such as in more complex actuator arrangements [29]; however, this is outside the scope of the current research. The overall performance of MPC depends heavily on the accuracy of the model used, and as such a more accurate dynamic model of the PAM would further improve the controller's performance.

Author Contributions: Conceptualization, D.F.B.; methodology, D.F.B.; software, D.F.B.; validation, D.F.B.; formal analysis, D.F.B.; investigation, D.F.B.; resources, D.F.B.; data curation, D.F.B.; writing—original draft preparation, D.F.B.; writing—review and editing, D.F.B. and S.Q.X.; visualization, D.F.B.; supervision, S.Q.X.; project administration, S.Q.X.; funding acquisition, D.F.B. and S.Q.X. All authors have read and agreed to the published version of the manuscript.

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Article

Smart Bio-Nanocoatings with Simple Post-Synthesis Reversible Adjustment

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Abstract: Nanopatterning of signal-transmitting proteins is essential for cell physiology and drug delivery but faces challenges such as high cost, limited pattern variability, and non-biofriendly materials. Arthropods, particularly beetles (Coleoptera), offer a natural model for biomimetic nanopatterning due to their diverse corneal nanostructures. Using atomic force microscopy (AFM), we analyzed Coleoptera corneal nanocoatings and identified dimpled nanostructures that can transform into maze-like/nipple-like protrusions. Further analysis suggested that these modifications result from a temporary, self-assembled process influenced by surface adhesion. We identified cuticular protein 7 (CP7) as a key component of dimpled nanocoatings. Biophysical analysis revealed CP7's unique self-assembly properties, allowing us to replicate its nanopatterning ability in vitro. Our findings demonstrate CP7's potential for bioinspired nanocoatings and provide insights into the evolutionary mechanisms of nanostructure formation. This research paves the way for cost-effective, biomimetic nanopatterning strategies with applications in nanotechnology and biomedicine.

Keywords: protein-based coatings; self-assembly; active coating; smart materials; bio-mimetic; switchable; antireflective; eco-friendly

1. Introduction

Nanopatterning of signal-transmitting proteins plays an essential role in cell and tissue physiology and metabolism, from mechanotransduction [1–3] to context-dependent and reconfigurable signaling [4]. These properties of the nanopatterned surfaces are becoming even more critical with the increasing usage of nanostructured cargo microparticles [5,6]. Such microparticles are very promising in drug delivery, and enhancing their activity and specificity is essential for improving diagnostic and therapeutic outcomes [7–10]. Recent advancements in protein immobilization strategies, such as photocatalytically polymerized brushes [11], have led to spatially defined protein adhesion. Techniques that use noninvasive methods, such as light-sensitive photocatalysts and hydrogels [12–14] on surfaces with well-defined microenvironments, addressing challenges in cell biology and tissue engineering are crucial for developing biosensors and protein microarrays. Despite its potential,

nanopattern technology faces many challenges, such as manufacturing and designing nanoscale geometries [15], reproducibility, and high production costs [16] with relatively low yields [1]. Current technologies of surface nanopatterning and nanostructured particle synthesis are limited in the range of patterns they can produce and often rely on non-biocompatible materials and techniques [17]. Therefore, biomimicry offers a promising approach to developing more efficient, cost-effective, and bio-friendly nanopatterning strategies [18–20].

One key factor for arthropods' evolutionary success is their natural mastery of nanotechnology. The surfaces of most arthropods feature various nano-sized outgrowths, grooves, or depressions, which provide functional advantages such as improved vision or self-cleaning [21,22]. These enhanced properties arise from significant changes in the physical and chemical properties of materials at the nanoscale. It was shown that these nanostructures form by the self-assembly process of waxes and proteins. This discovery has enabled the reproduction of the self-assembly process in vitro [23], allowing for the modification of different surfaces. Since these nanocoatings are protein-based, they can be engineered to create surfaces involved in signaling or enzymatic activity. However, such modifications can disrupt the protein self-assembling ability [24]. To overcome this challenge, an alternative approach based on evolutionary analysis might provide a solution [18]. Based on these initial observations, we conducted a large-scale screening of the Coleoptera order to identify trends and patterns of potential solutions.

2. Materials and Methods

Coleoptera samples were obtained from the Zoological Museum of Lomonosov Moscow State University (Moscow, Russia). The cuticles from a minimum of 3 animals of each species were carefully excised. The samples were then mounted on coverslips using double-sided adhesive tape. Samples were stored in closed Petri dishes, and it was shown that they are stable in air for at least 4 years [23]. For the wettability tests, some samples were washed in Milli-Q water while rubbing with a cotton swab.

2.1. Atomic Force Microscopy (AFM)

Atomic force microscopy (AFM) was performed using multiple instruments and operational modes. Measurements in tapping mode were conducted with a Shimadzu SPM-9600, using NS16 cantilevers (tip radius 8 nm, frequency 190 kHz, length 225 μm) (NT-MDT, Moscow, Russia). Imaging in contact mode (0.3 nN) was performed using an XE-100 microscope (Park Systems, Suwon, Republic of Korea) using PPP-NCHR cantilevers (tip radius of curvature < 10 nm, length 125 μm) (NanoWorld, Neuchâtel, Switzerland). Adhesion Force Microscopy was performed by the NanoWizard 4XP (Bruker, Billerica, MA, USA) in QI mode using Scansyst-air cantilevers (tip radius 2 nm, frequency 70 kHz, length 115 μm) (Bruker). The Gwyddion software version 2.67, developed by the Department of Nanometrology at the Czech Metrology Institute (Brno, Czechia), was employed to visualize and quantify the acquired data.

2.2. Simulations

Simulations were performed using the following approach: the surface roughness was characterized in terms of the root means square (RMS) roughness, σ defined by $\sigma = \sqrt{a^{-2} \int_{x=-\frac{a}{2}}^{\frac{a}{2}} \int_{y=-\frac{a}{2}}^{\frac{a}{2}} h(x,y)^2 dy dx}$, where a is the side length of the square sample, and $h(x,y)$ is the function describing the height of the sample surface at (x,y) . For the considered range of wavelengths λ (300 nm to 800 nm), we find that the ratio σ/λ is bounded by approximately 0.1 for all AFM scans, which is a sufficiently small value to justify the use of the total integrated scatter (TIS) formula for reflectance at normal incidence [25],

$R(\lambda) = \left| \frac{n_{\text{substrate}} - n_0}{n_{\text{substrate}} + n_0} \right|^2 \exp(-16\pi^2\sigma^2/\lambda^2)$. Here, $n_{\text{substrate}} = 1.6$ [26] and $n_0 = 1$ are the refractive indices of the substrate material and of free space, respectively, and the squared fraction in the reflectance formula is the diffraction efficiency for a smooth air–substrate interface at normal incidence.

2.3. Wettability Test

To assess surface wettability, Milli-Q water droplets were dispensed using a capillary attached to a micromanipulator (MN-4, MMO-203, Narishige, Tokyo, Japan). The behavior of the water droplets was observed under an inverted microscope (Axiovert 40 C, Zeiss, Oberkochen, Germany). Images of the droplets were captured using a digital camera, and the contact angles of the droplets were measured on both sides using Gwyddion software version 2.67.

2.4. Mass-Spectrometry

Corneal samples for proteomic analysis were prepared by excising the eyes from the heads of mature adult beetles using a scalpel. The excised eyes were scraped from the inner surface and washed three times with Milli-Q water. The cleaned corneal samples were then incubated at 95 °C in loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1% β -mercaptoethanol, a trace amount of bromophenol blue) for 2 h, as previous experiments [23,27] have shown that this time is acceptable to wash a sufficient amount of the material from the lenses but not long enough to lead to protein degradation. The samples were analyzed using SDS-PAGE, and the bands corresponding to the protein of interest were excised and sent to the Protein Analysis Facility of the University of Lausanne (Lausanne, Switzerland). Proteins were in-gel digested with trypsin, and peptides were analyzed by nanoLC-MSMS using an easy-nLC 1000 liquid chromatography system (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Protein Purification

For protein purification, the gene sequence encoding the CP7 protein with an RGS-His tag at the N-terminus was custom-synthesized and cloned into a pET-28a(+) vector (Synbio Technologies, Monmouth Junction, NJ, USA). The plasmid was transformed into the *E. coli* BL21(DE3)pLysS strain for recombinant protein expression upon induction with 1 mM IPTG. The bacterial mass was lysed by a cell press (Constant Systems, Daventry, UK). The His-CP7 was purified using the HisPur Ni-NTA resin (ThermoFisher Scientific, Waltham, MA, USA) with prewashing using a 2 M Urea solution to exclude the possibility of protein–protein binding. This was followed by purification using the Äkta liquid chromatography system using the Superdex® 200 Increase 10/300 GL column (Sigma-Aldrich, St. Louis and Burlington, MA, USA).

2.6. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE), proteins were mixed with Laemmli buffer, boiled at 95 °C for 5 min, and loaded onto an 18% acrylamide gel. For Coomassie staining, the gels were washed twice with water for 5 min, then incubated in colloidal Coomassie stain solution for 2 h. Destaining was performed overnight in water.

2.7. Western Blotting

For Western blotting, proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Chicago, IL, USA) using a wet transfer

system (Bio-Rad, Hercules, CA, USA) at 100 V for 3 h. The membrane was then blocked in 5% low-fat milk in TBS for 1 h, which is a standard procedure used to reduce the background signal on the membrane, followed by incubation with an anti-RGS-His primary antibody (mouse, 1:1000 dilution, Qiagen, Hilden, Germany, cat. no: 34650). HRP-conjugated secondary antibodies were used for detection via enhanced chemiluminescence in a Fusion FX6 Edge system (Vilber, Collégien, France).

2.8. Wax Emulsion Preparation

Wax emulsions were prepared by adding 4 g of lanolin wax (Sigma-Aldrich, St. Louis and Burlington, MA, USA) to 40 mL of 10% SDS solution and sonicating the mixture at 80 °C for 2 h in a water bath (AL 04-04, ThermoFisher Scientific, Waltham, MA, USA) [28,29]. The emulsified solution was left to stand at room temperature for 24 h, after which the upper layer enriched in wax nanodroplets was collected, diluted tenfold in $1 \times$ TBS, and incubated for 48 h. After incubation, only the lower layer of the solution was collected and diluted in TBS until it reached a concentration equal to 0.8 mg/mL (for CD) or 0.4 mg/mL (for artificial coatings). The final verification was carried out using a ZetaSizer Nanoseries (Malvern Pananalytical, Malvern, UK) analyser, using DTS 1070 cells (Malvern, UK) according to the manufacturer's protocol.

2.9. Circular Dichroism

Circular dichroism measurements were performed in the Department of Organic Chemistry of the University of Geneva using the Jasco J-815 circular dichroism spectrometer with strain-free QS quartz 1 mm path-length cuvettes (Jasco, Easton, MD, USA). The His-CP7 protein was used at 0.23 mg/mL in 25 mM potassium phosphate buffer, pH 7.5.

2.10. ThT Fluorescence Measurement

The ThT fluorescence levels were measured by the Multimode plate reader Hidex Sense (Hidex, Turku, Finland) from the plate (microplate 96 well, Greiner Bioone, Kremsmünster, Austria) bottom with excitation of 355 ± 40 nm and emission of 535 ± 20 nm [30]. The final concentrations were 30 μ M ThT (Sigma-Aldrich, St. Louis and Burlington, MA, USA, #T3516), 0.12 mg/mL proteins, and 0.004% SDS. Before measurement, ready solutions were incubated for 10 min at room temperature.

2.11. Nanocoating Generation

Nanocoatings were created by applying a mixture of 5 μ L His-CP7 (0.65 mg/mL in TBS) and 15 μ L of lanolin (0.4 mg/mL in TBS) emulsion onto a 1 cm² glass coverslip. The applied mixture was dried, rinsed with water, and re-dried. This process was repeated twice to ensure proper coating. Switching of corneal or artificial nanocoatings was performed by applying a solution of 1 μ L His-Reinin (0.5 mg/mL in TBS), 2 μ L of lanolin (0.4 mg/mL in TBS) emulsion, and 16 μ L of TBS on surfaces. The applied mixture was dried, rinsed with water, and re-dried.

2.12. Statistical Analysis

Statistical analysis was performed using GraphPad Prism software Version 10.0.3.

3. Results

3.1. Two Types of Nanostructures and Their Functionality

Nanoscale analysis of the corneal structures in Coleoptera revealed variations in nanocoatings among different specimens [24]. Atomic force microscopy (AFM) analysis of the superfamily Tenebrionoidea showed that the commonly observed dimpled nanocoatings are sometimes modified into maze-like or nipple-like structures (Figure 1a–d) [21,31].

This duality was observed in species such as *Melandrya caraboides*, *Oedemera virescens*, *Schizotus pectinicornis*, *Rhampholyssa steveni*, *Mylabris calida*, and the *Omophlus* sp. (Figure 1a–c). These structures resemble the surface textures found in butterfly eyes [32]. Such modifications are thought to enhance antireflectivity, which is considered the primary function of corneal nanocoatings [33]. The reduction in reflected light is attributed to the formation of a refractive index gradient by nanostructures smaller than the wavelength of the light. The effective refractive index at any depth of such nanocoating is the weighted sum of the refractive indices of the materials present. For instance, if half of the coating consists of lens material and the other half air, the effective refractive index will be between those of air and the lens material [34,35]. Light passes through this nanostructured coating as if through a medium with a smooth gradient of refractive index. Numerous theoretical and experimental investigations into nanostructures have shown that taller structures tend to exhibit superior antireflective properties [35,36], suggesting that the nipple-like and maze-like structures provide better antireflective properties. However, these coatings are also more prone to damage, which can compromise their functionality. This creates a dilemma: how to maximize efficiency while minimizing the risk of environmental harm. For example, flying insects like butterflies can support higher nanopillars [37], as they are less susceptible to collisions compared to crawling insects [38].

Due to the uneven distribution of these modified nanostructures, we could not experimentally verify this hypothesis. To address this limitation, we decided to simulate the light reflectance using the total integrated scattering method. The results showed a significant reduction in light reflectance for both tested insect specimens (Figure 1e).

Nanocoatings have a significant effect on the wettability of the materials they cover. There are two primary models that describe this behavior: the Wenzel and the Cassie–Baxter models. The Wenzel model assumes that the entire surface is completely wetted, while the Cassie–Baxter model posits that gas becomes trapped in the cavities created by the surface’s roughness. Experimental data reveal that the actual interactions between solid, liquid, and gas often fall somewhere between these two models, influenced by the morphology of the structures involved [38,39]. Usually, structures smaller than 50 nm exhibit wetting behavior as described by the Wenzel model, which largely depends on their chemical composition [26,40]. Since in the Wenzel state the antireflective and antiadhesive functionalities of corneal nanocoatings are typically mutually exclusive [23], we tested the hydrophobicity of these surfaces. As expected, the modification made the surface hydrophilic, as indicated by a drastic decrease in the contact angle to approximately 70°, a characteristic value for a standard nipple array structure [24]. However, the nature of this modification was temporary, suggesting that it could be washed away along with other contaminations if needed [41]. Indeed, gentle washing with water, combined with light rubbing, partially restored the hydrophobicity (Figure 1f).

3.2. Switching Mechanism

Given the rigid nature of the corneal surface, we proposed that the structural changes could result from a specific form of directed contamination. As previously shown, grooves of nanocoatings differ in nature compared to the hills [42,43]. With an ester-based chemical composition, the grooves exhibit higher adhesion, which is reflected in their better wettability [23,40,44]. To confirm this, we directly measured the adhesion of *R. steveni* corneal samples by using AFM. Indeed, the results revealed that the composition and, consequently, the adhesion of the nanocoating is not uniform. The maximal adhesion force was colocalized with topographical dimples (Figure 2a,b). We suggested that these dimples in the corneal coating could direct the next step of the assembly, facilitating the growth of new, secondary nano-protrusions on the top of the hollows and dimples (Figure 2c). These

secondary nanostructures are less stable and can be easily washed away, but, at the same time, they might enhance the antireflective properties of the dimpled structures.

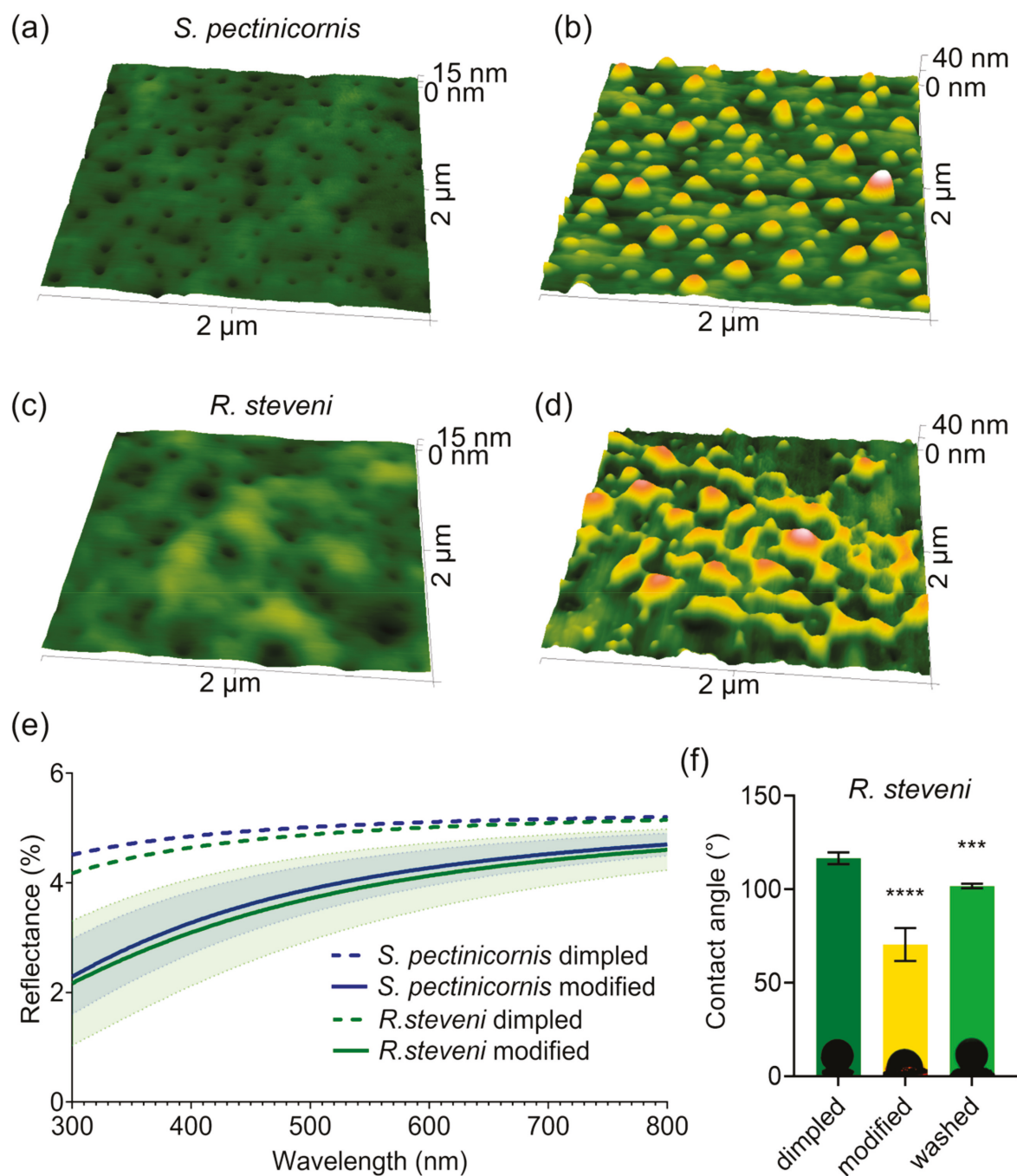


Figure 1. Unmodified and modified corneal nanocoatings. (a,d) Unmodified (a,c) and modified (b,d) corneal nanocoating AFM topography scans of *S. pectinicornis* (a,b) and *R. stevensi* (c,d). (e) Simulated reflection spectra of the investigated surfaces. Clear improvement of the antireflectivity by modification is visible. Data are shown as mean (lines) $\pm$ range (semi-transparent). (f) Water contact angles of the eye surface from *R. stevensi* unmodified (dimpled) samples, modified samples, and modified samples after cleaning. The bar and error bars indicate the mean $\pm$ SD; $n = 4$. Statistical significance is tested by a two-tailed t -test (“****” $p < 0.001$, “****” $p < 0.0001$). The representative images of the droplets are shown as bar inserts.

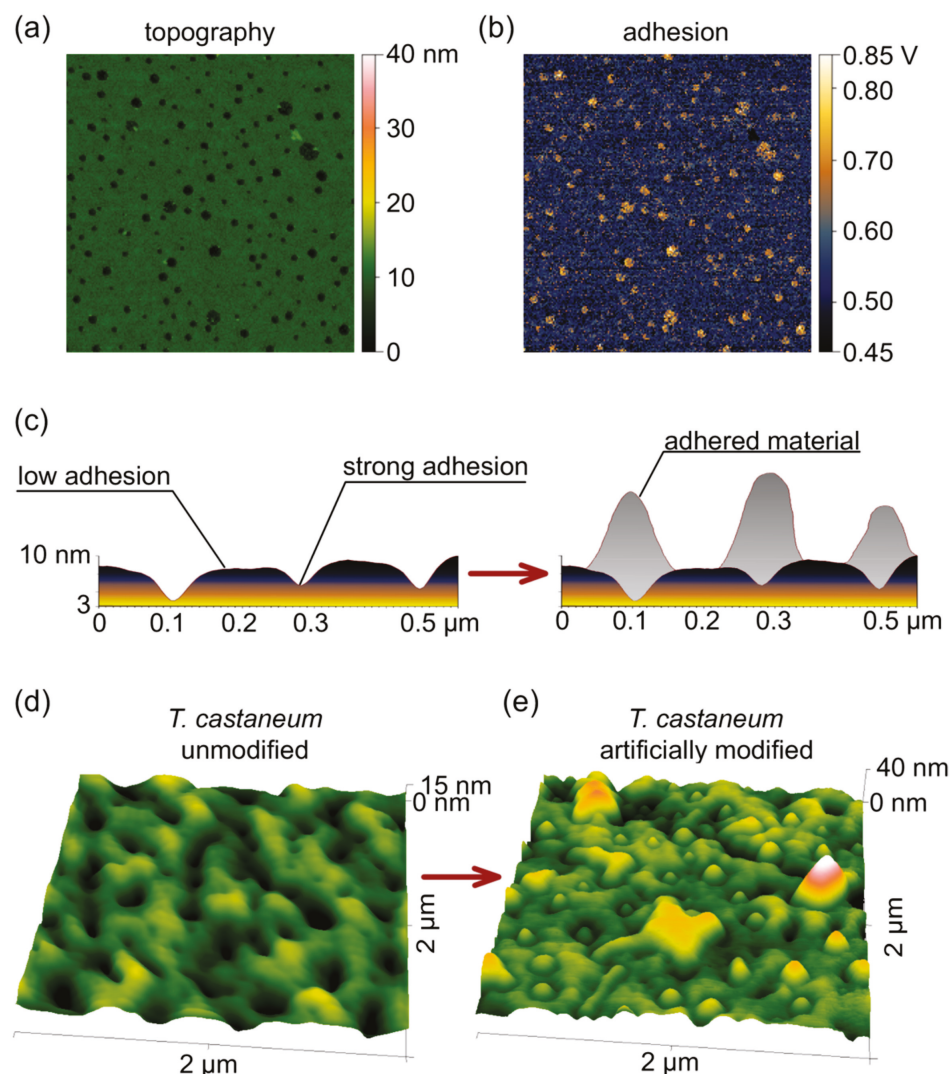


Figure 2. The uneven adhesion and topography of Tenebrionoidea corneal nanocoatings lead to area-specific deposit aggregation. (a) AFM topography scan of the *R. steveni* cornea. (b) The same area, but showing adhesion data on the nanoscale. Clear topography dependence is visible. Scans are $2 \times 2 \mu\text{m}$. (c) Proposed mechanism of a specific material aggregation over the most adhesive areas. Heights and shapes are based on AFM topographical data of *R. steveni* corneae. (d) *T. castaneum* corneal nanocoating AFM topography scan. (e) After applying a self-assembling wax–protein mixture, the same sample shows the appearance of protrusions.

The switching mechanism might involve the secretion of a specific adhesive material. For example, the same self-assembling protein/wax mixture could be applied on a surface, as was shown for whip spiders [17]. To test this hypothesis, we artificially coated the dimpled corneal cuticle of *Tribolium castaneum* (Tenebrionoidea) with a diluted solution of wax and protein. This solution was able to form protrusions on top of existing nanodimples (Figure 2d,e).

3.3. Reverse Engineering

In our previous work, we developed a workflow that enabled the identification of Retinin as the self-assembling morphogen in the corneal nanocoatings of *Drosophila* [23]. Using the same approach, we analyzed the corneae of other insects with fully sequenced genomes, including the silk moth *Bombyx mori* [27], the common Mormon butterfly *Papilio polytes* [26], and the mosquito *Anopheles gambiae* [45]. Given that the red flour beetle (*T. castaneum*), a standard representative of the Tenebrionoidea superfamily, also has a

fully sequenced genome [46], we investigated its corneal proteomics (Figure 3a). Despite challenges in the material collection [47], we successfully identified the primary small corneal protein as TC003109, also known as cuticular protein 7 (CP7).

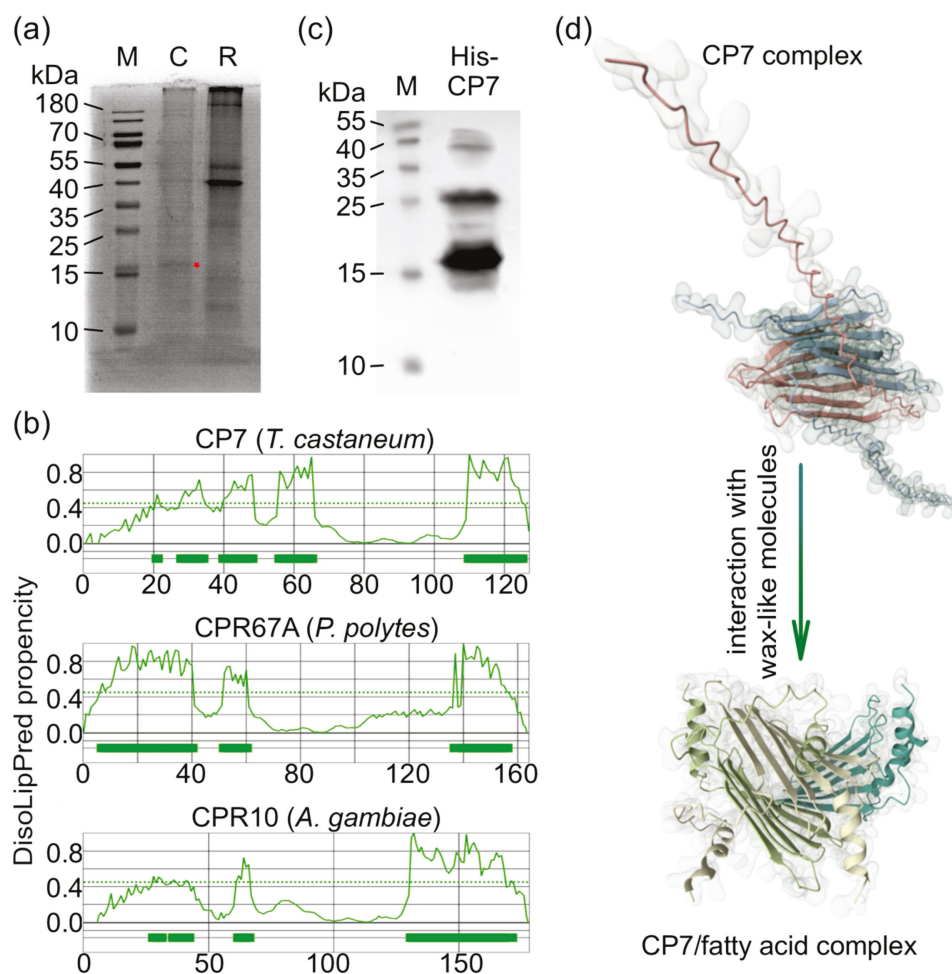


Figure 3. CP7 is a possible building block of Tenebrionoidea-like dimpled corneal nanocoatings. (a) SDS-PAGE analysis of corneal (C) and retinal (R) samples with CP7 protein band, used for the mass-spectrometry analysis, indicated by the red star. (b) DisoLipPred algorithm predicts extensive disordered lipid-binding regions in CP7, CPR67A, and CPR10. Thick lines show disordered lipid-binding regions. (c) Western blot of purified RGS-His-CP7 shows the presence of stable oligomers in solution. (d) The potential complexation of CP7 subunits (shown by colors) in the solution and their assembly into a more compact and likely stable complex forced by interaction with fatty acids. Predicted by AlphaFold (<https://alphafoldserver.com>, accessed on 23 January 2025).

CP7 from *T. castaneum*, Retinin from *D. melanogaster*, CPR10 from *A. gambiae*, CPR67A from *P. polytes*, CPR150 and CPR19 from *B. mori* are all small secreted proteins that lack sequence homology with one another and are predicted to have extensive intrinsically disordered regions (IDRs). The disordered nature of Retinin and CPR67A has been confirmed in our previous work [23,26]. A critical feature enabling these proteins to participate in nanocoating formation is the lipid-binding capacity of their IDRs. Indeed, many IDRs are known to interact with lipids [48], and lipid-binding activity may be a common characteristic of IDRs and IDR-containing proteins [49–54]. Not only corneal proteins but also other IDR-containing proteins have been shown to adopt an induced-fit conformation upon interacting with lipids [54]. The formation of high-molecular-weight condensates composed of IDR proteins and lipids has also been experimentally observed [49], suggesting that it could be the first step in the nanostructure self-assembly process [23]. Analysis

using the DisoLipPred algorithm [50] confirmed the presence of lipid-binding regions in CP7, CPR67A, CPR10, revealing a similar organization of these regions across the proteins (Figure 3b). Based on these findings, we propose that CP7 is responsible for the formation of Tenebrionoidea-like dimpled corneal nanocoatings. However, recombinant CP7 protein was found to behave differently than previously investigated CPR10 and CPR67A. Size-exclusion liquid chromatography, Coomassie-stained SDS-PAGE, and Western blot analyses of recombinant His-CP7 showed that this protein exists as dimers and oligomers, even under denaturing conditions (Figure 3c). Previous data on protein purification using this method [24] and an additional step of washing Ni-NTA beads exclude the possibility of contamination. Indeed, *in silico* [55] analysis indicated that CP7 dimers could form in solution without any ligands. Adding myristic acid to this simulation resulted in additional folding and the assembly of a more compact complex (Figure 3d).

Biophysical analysis of CP7 confirmed its distinct properties, differentiating it from the group of previously identified nanocoating-forming proteins. Circular dichroism analysis has shown that CP7 is initially folded, and its interaction with wax alters its tertiary structure but not as drastically as in the case of other Retinin-like proteins (Figure 4a). At the same time, we observed the absence of β -sheets capable of binding ThT [56] in the protein without a wax, which appeared gradually as the wax concentration increased. However, the intensity of the interaction with ThT was even lower than that for the least active CPR67A protein (Figure 4b).

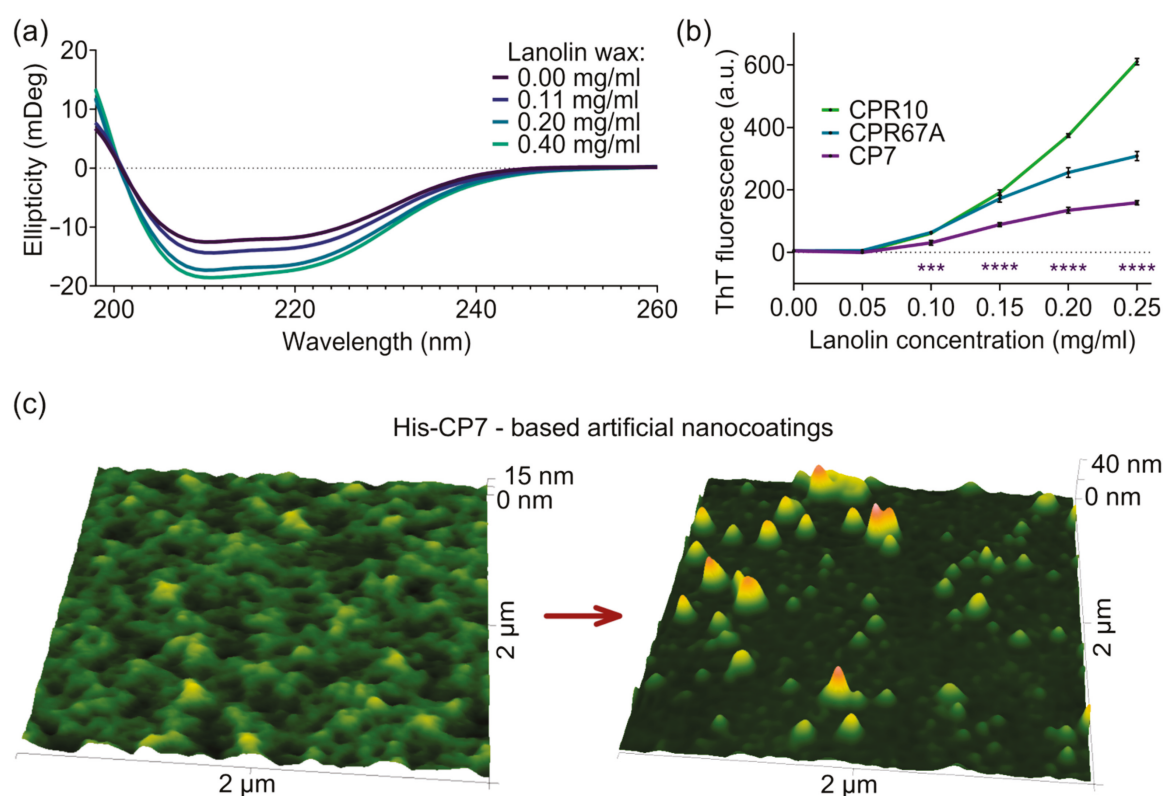


Figure 4. CP7 biophysical properties allow for the self-assembly of artificial nanocoatings. (a) Circular dichroism spectra of RGS-His-CP7 admixtures with different concentrations of the lanolin wax emulsion show protein folding. (b) ThT fluorescence measurements of proteins mixed with a lanolin emulsion at different concentrations. Data are shown as mean $\pm$ s.d, $n = 4$. Statistical significance compared to the control without wax emulsion was determined using a two-tailed *t*-test (**** $p < 0.001$, ***** $p < 0.0001$). (c) Nanocoatings produced by using His-CP7 with the lanolin wax resemble corneal nanocoatings of *T. castaneum* (left) and can be modified by applying a self-assembling wax-protein mixture (right).

Given the unique properties of CP7, we proceeded to test the self-assembly of the CP7/lanolin wax mixture on the glass. Using the protocol that produced nipple array nanocoatings in the case of CPR10 and CPR67A, we successfully reproduced the dimpled pattern with the CP7 protein. Additionally, secondary coating of the dimpled surface with a diluted wax and protein solution resulted in the formation of protrusions on top of existing nanodimples, similar to the corneal samples (Figure 4c).

4. Discussion

The evolution, origin, and development of modifiable nanocoatings on the insect cuticle remain enigmatic. While all studied beetles belong to the same superfamily of Tenebrionoidea, their classification provides little insight into the specific evolutionary pressures of mechanisms behind the emergence of this unique adaptation. Furthermore, the ecological diversity of these beetles, both as adults (imago) and larvae, varies significantly among the four studied species and complicates the search for a unifying driver. Adults of *O. virescens* inhabit meadows, ranging from forested areas to the steppe zone, and are typically found on flowers [57]. In contrast, *R. steveni* occupies sandy and floodplain areas in semi-desert and desert regions, and adults are also commonly found on flowers [58]. *M. caraboides* and *S. pectinicornis* are forest-dwelling species, but their specific habitats differ [59]. Adults of *S. pectinicornis* are primarily found on tree trunks, particularly birch [60], while *M. caraboides* reside on trunks and beneath the bark of dead trees [61]. These ecological variations in these species do not suggest a specific evolutionary path driven by specific selective pressures. It is possible that these nanodimpled coatings are primordial and appeared earlier than the Tenebrionoidea superfamily, as similar structural modifications have been observed on the surface of underwater amphipods [62]. Their bi-functionality possibly provided just the right ecological trade-off to persist in the lineages of arthropods.

There are three potential mechanisms by which insects modify their corneal coatings. The first involves the use of naturally occurring impurities deposited on the surface from various sources. However, this mechanism has significant limitations, as the physical properties of these materials are inherently unpredictable. The second mechanism, discussed in the results, involves the secretion of a mixture capable of self-assembly on the dimpled surface. We suggest that such a mixture can consist of compounds similar to those that are assembled by the reaction–diffusion mechanism to the initial nanocoatings. And reversibility is then easy to achieve by limiting the binding of newly appearing nanostructures to the surface. While such behavior has been observed in whip spiders [63], there are currently no reports or observations of beetles distributing secretion material over the surface of their eyes. It is also possible that this secretion is produced by hypodermal cells within the eye cuticle. The secretion would then exit onto the eye's surface through pore channels. Although such channels have been identified in some hexapods [64] and in olfactory organs [31], no evidence of pore channels has been reported for insect corneas [37,65,66].

The antireflective functionality of these modifications can exist only in the case of the dimpled pattern. Appearing protrusions double the distance from hollows to the peaks, which is the main factor in decreasing light reflectance [35,36]. In contrast, the nipple array would only be flattened by such modifications, as was shown for *D. mojavensis* and *P. delalandei* [23,26]. However, the presence of such flattened modified nanocoatings lets us suggest the existence of additional functionality, such as anti-contamination properties against waxy particles or against dust, as shown in the case of whitefly [67].

The newly identified protein CP7 differs from all other Retinin-like proteins, possessing a stronger tendency for the formation of oligomers and much weaker activation by wax simultaneously. The higher self-activation rate and lower activation by the inhibitor could

explain this phenomenon. These data not only provide a way to detect the exact molecular mechanism of protein-based nanostructure assembly but also allow for the combination of different building blocks, as demonstrated in this article.

5. Conclusions

This study highlights that the dimpled pattern plays a crucial role in antireflective properties, reducing light reflectance, and may serve additional purposes, such as anti-contamination, due to its ability to guide the secondary reversible coating by its specific topographical and adhesive characteristics.

The artificially engineered structures we developed closely replicate the physical properties observed in natural systems, demonstrating the feasibility of their synthesis under mild and controlled conditions. This innovative approach offers significant advantages, particularly for applications where conventional treatment methods are unsuitable, such as the fabrication of contact lens surfaces, living tissues, or prosthetic devices. For these surfaces, the limitations of coatings, such as relatively weak binding to the substrate and low wear resistance [45], are minor.

These synthetic coatings consist of a wax-enriched base layer that provides durability, complemented by a protein-enriched layer on the top. The unique construction of these coatings opens up possibilities for a wide range of applications, extending their utility beyond the original purpose. For example, proteins in the modification layer can be engineered with peptides or short proteins and then nanopatterned through a self-assembly mechanism. This approach enables the precise programming of structure density and size, which has been shown to enhance protein stability [68]. Surfaces with pre-patterned active proteins could lead to the development of novel sensors as well as advancements in tissue engineering [69] and the study of cell behavior [70].

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biomimetics10030163/s1>, Table S1: Mass-spectrometry analysis of *T. castaneum* corneal proteins.

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Article

Mechanics of Bio-Inspired Protective Scales

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Abstract: Natural armors found in animals like fish and armadillos offer inspiration for designing protective systems that balance puncture resistance and flexibility. Although segmented armors have been used historically, modern applications are hindered by a limited understanding of their mechanics. This study addresses these challenges by presenting two novel bio-inspired scale structures with overlapping and staggered configurations, modeled after the elasmoid designs found in fish. Their shapes differ significantly from other artificial scales commonly described in the literature, which are typically flat. Instead, these scales feature a support that extends vertically from the substrate, transitioning into an inclined surface that serves as the protective component. Finite element method tests evaluated their performance in puncture resistance and flexibility. The results showed that one type of scale provided better puncture resistance, while the other type offered greater flexibility. These findings highlight how small geometric variations can significantly influence the balance between protection and flexibility. The results offer new insights into the mechanisms of natural armor and propose innovative designs for personal protective equipment, such as bulletproof vests, protective gloves, and fireproof systems. The finite element simulations employed to test the protective systems can also serve as valuable tools for the scientific community to assess and refine designs.

Keywords: scales; protective systems; puncture-resistant; biomimetics

1. Introduction

There are multiple examples of animals equipped with protective systems. In any type of natural application, the protective structures are mainly used to defend the epidermis of the animal in question from the attack of other animals and from natural environmental adversities that it is facing. In reality, often, these dermal protective systems also have secondary functions, aside from simple protection of the body. In fact, they are multifunctional, ensuring, for example, reduction in hydrodynamic friction in fish, coloring by camouflage in some reptiles, luminescence, and regulation of temperature. Flexible natural armors are attracting increasing attention for their unique combinations of hardness, flexibility, and lightness. The extreme contrast of the rigidity between hard scales and surrounding soft tissues inspires the design of biomimetic protection systems that are simultaneously difficult to drill and at the same time flexible. In particular, it has been seen that fish have protective structures formed by small overlapping and staggered small elements, scales, which accentuate the protective effects, without influencing in any way the weight and the ability to move. Thus, in the biomimetic field, research was carried out to create protective solutions inspired by the hierarchical structures in scales seen in nature [1–41]. It is believed that these can be applied by humans to improve the protective systems currently in use, such as gloves, bulletproof vests, or other.

A review of flexible dermal armor for the protection of fish, reptiles, and mammals has been delivered by Yang et al. [1]. Another review by Naleway et al. [2] presents the basic building blocks of the structural biological materials and a variety of protective strategies in marine organisms, with a focus on their structure and mechanical properties and a discussion on the bio-inspired potential of these biological materials. Natural protective systems are distinguished by their hierarchical structures, which integrate hard and soft components to optimize mechanical performance. Fish scales, for example, exhibit overlapping designs with mineralized layers and collagen fibers that provide both flexibility and puncture resistance [1–8,20,30]. Ganoid scales, such as those of alligator gar, demonstrate unique anisotropic properties due to their ganoine layer and bony foundation [3,4,19]. The elasmoid scales of teleost fish, like carp, feature Bouligand structures that enhance toughness and energy dissipation [3,4,20]. Marine organisms such as chitons and seahorses also display remarkable protective adaptations. Chiton girdle scales offer flexibility and protection through their tessellated arrangement [36], while the articulated armor of seahorses balances flexibility and resistance to crushing [22]. Shark skin, characterized by denticles, combines hydrodynamic efficiency with mechanical resilience [29]. Osteoderms in reptiles, such as alligators and armadillos, further highlight the role of mineralized structures in providing mechanical support and thermal regulation [15,16]. Pangolin and turtle scales offer insights into hierarchical design principles where overlapping structures enhance both flexibility and damage resistance [17,18].

The mechanical properties of natural protective systems are as diverse as their structures. Studies on pangolin scales reveal their ability to resist deformation through overlapping keratinous plates [17,39]. Fish scales, such as those of striped bass and arapaima, demonstrate nonlinear flexural behavior that supports locomotion and energy absorption [27,28]. Armadillo and turtle shells, composed of osteoderms, achieve toughness and puncture resistance through their hierarchical and mineralized structures [16,18]. The dynamic response of fish scales under high strain rates has also been explored, revealing enhanced puncture resistance due to the layered microstructure [21]. Seahorse armor, with its segmented bony plates, provides insights into fracture resistance and prehensile capabilities [27]. Similarly, the mechanics of striped bass skin have been linked to the synergistic behavior of scales and underlying collagen layers, which act as an exotendon during locomotion [28]. Studies on the mechanical response of biomimetic materials, inspired by these systems, highlight the potential to replicate their toughness and flexibility in synthetic designs [10,27]. The balance between hardness and compliance observed in these systems underpins their ability to provide protection without compromising mobility, a principle that has been extensively applied in bio-inspired materials research.

Advanced computational approaches have been instrumental in understanding the mechanics of natural protective systems. Finite element models have been used to simulate the deformation of fish scales and segmented armors, revealing the effects of geometry and material properties on their mechanical behavior [8,31,32]. Discrete element methods have further elucidated the interaction between individual scales and soft substrates, providing insights into their anisotropic response [27,32]. Frictional effects in biomimetic scales have been analyzed to understand their role in nonlinear mechanical behavior, while coupled bend–twist mechanics have demonstrated the complex kinematics of scale systems [34,35]. Analytical models, combined with experimental data, have helped predict the dynamic response of fish scales under various loading conditions, offering guidelines for optimizing bio-inspired designs [8,14]. Further computational studies have delved into the role of scale morphology and interlocking patterns on puncture resistance and energy absorption. For instance, models of elasmoid fish scales have shown how interscale friction and overlapping arrangements enhance mechanical performance under dynamic loading conditions [13,26].

These insights provide a foundation for the design of advanced flexible composites and protective materials.

The translation of natural design principles into synthetic materials has led to groundbreaking advancements in bio-inspired armor and flexible composites. Three-dimensional printing has enabled the fabrication of bio-inspired structures, replicating the overlapping and hierarchical arrangements of fish scales to enhance mechanical performance [14,40]. Segmented ceramic armors, inspired by osteoderms and fish scales, have been developed to balance toughness and flexibility in protective applications [4,39]. Biomimetic shark skin and fish scales have found applications in hydrodynamics and energy absorption, demonstrating the versatility of bio-inspired designs [13,29]. The unique morphology and mechanical adaptability of these systems have also inspired innovations in robotics, aerospace, and wearable technologies [25,26]. Additionally, studies on pangolin and armadillo scales have led to the development of materials with enhanced resistance to penetration and impact, providing insights into multifunctional armor systems [15,17].

Fish have protective systems designed to protect them both from attacks by other fish, or predators and to reduce resistance to hydrodynamic advancement. These systems are formed by hierarchical structures comprising scales of resistant materials placed on a soft substrate, that is, the skin of the fish. The scale systems present in fish vary both according to the characteristics of the individual scales in terms of shape, size, and material and according to their arrangement on the soft substrate. Fish have protective structures with overlapping scales; however, the degree of overlap is a variable factor in the transition from one species to another. Among the main factors that influence the behavior of the coating in terms of protective capacity and motion flexibility, there is the type of scales a fish has. There are four types of scales, namely placoid, ganoid, cosmoid, and elasmoid (which have two sub-categories, ctenoid and cycloid). Figures 1 and 2 show several types of scales, also presenting the typical degree of overlap that can be found with fish having this type of scale.

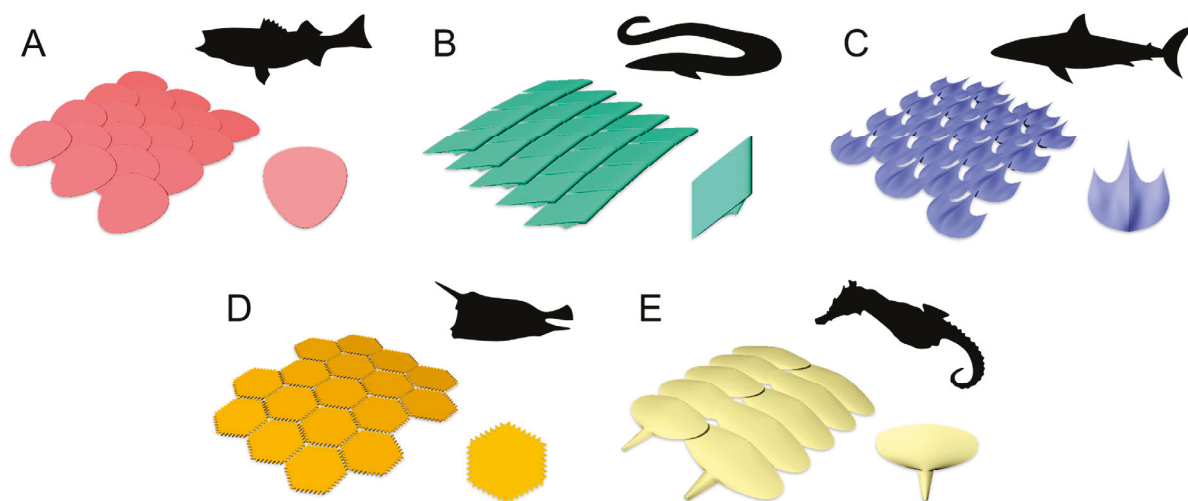


Figure 1. Representative schematics of common protective armors among living fishes. (A) Overlapping elasmoid scales common among most teleosts, such as striped bass; (B) interlocking ganoid scales common among gars and bichirs; (C) partially imbricated placoid scales common among sharks; (D) tessellating carapace scutes common among boxfishes; (E) interlocking and overlapping bony plates common among seahorses and related syngnathid fishes. Reproduced with permission from M. M. Porter et al., *J. of the Mech. Behavior of Biomedical Mat.*, 73 (2017) 114–126. ©2017, Elsevier [3].

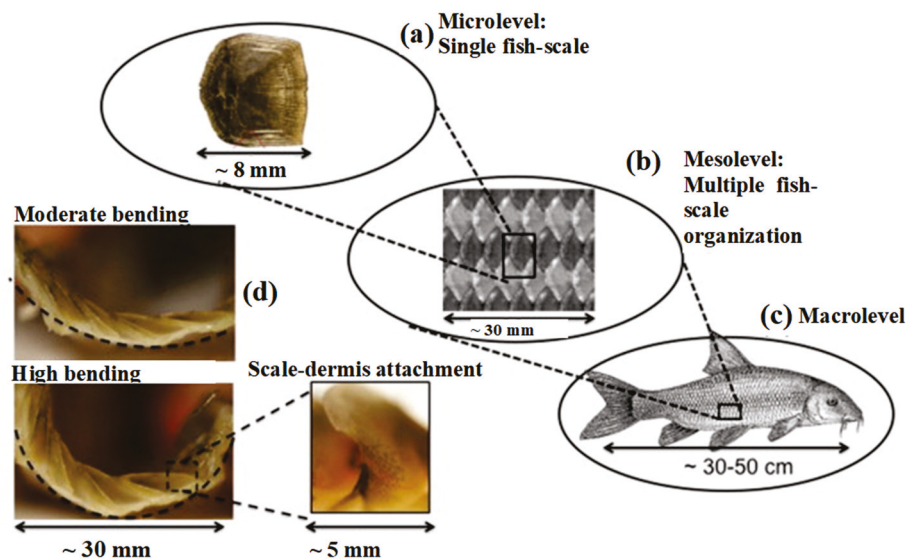


Figure 2. Structure of fish skin from the microlevel (single fish-scale) (a), to the mesolevel (b), to the macro-level (full skin) (c). Bottom left figures, (d), Subfigures (d) illustrate the deformation of fish skin during bending, highlighting a pronounced rotation of individual scales. Reproduced with permission from F. J. Vernerey et al., *Int. J. of Solids and Structures*, 51 (2014) 274–283. ©2017, Elsevier [8].

In recent years, strong research has been carried out on protective scale structures to identify possible biomimetic solutions inspired by fish. In particular, synthetic models were created, reproducing the hierarchical structure in scales, and mechanical tests were subsequently performed. A review of recent studies that use 3D printing techniques to investigate the biomechanics of armored fishes specifically was provided by Porter et al. [3]. To understand the behavior of the structure, these tests were performed with the variation of some characterizing parameters, such as scale material, material of the substrate representative of the skin, scale geometry, size of the scales, and degree and type of overlap of the scale structure. Martini et al. [4] used 3D printing to fabricate arrays of scales with increasingly complex geometries and arrangements, from simple squares with no overlap to complex ganoid-scales with overlaps and interlocking features. Stiff polymer (ABS) scales were glued onto a much softer substrate (polyurethane) with a thickness of 3 mm; then, they performed puncture tests and flexural tests on each of the 3D-printed materials and reported the puncture resistance and compliance characteristics of each design. In another study, Martini et al. [5] studied flexible bio-inspired armor based on overlapping ceramic scales, high purity alumina, and a soft silicone elastomer for the membrane. The fabrication combined laser engraving and a stretch-and-release method, which allowed for fine tuning of the size and overlap of the scales; they found out that compared to a continuous layer of uniform ceramic, the fish-scale like armor is not only more flexible but also more resistant to puncture and more damage-tolerant. The proposed armor is also about ten times more puncture-resistant than soft elastomers, making it a very attractive alternative to traditional protective equipment.

Some experiments were performed also directly on the fishes. Szewciw et al. [6] investigated the flexural response of whole teleost fish and how scales affect global flexural stiffness. A bending moment was imposed on the entire body of a striped bass (*Morone saxatilis*). Imaging was used to measure local curvature; it was found that the flexural stiffness is the highest in the thick middle portion of the fish and lowest in the caudal and rostral ends. The flexural response is nonlinear, with an initial soft response followed by significant stiffening at larger flexural deformations. In another work, Szewciw et al. [7] explored the possible additional role of the skin in fish undulatory lo-

comotion by examining the structural and mechanical properties of the dermal stratum compactum layer of striped bass (*Morone saxatilis*) skin. The structure, mechanical response, and function of stratum compactum was investigated by combining several methods: optical microscopy and histology, tensile tests on descaled skin specimens in different anatomical locations and orientations, puncture tests, and flexural tests on whole fish with disruption of the s. compactum.

To obtain a better understanding of the scale mechanics, the finite element method (FEM) was used to perform parametric studies. Vernerey et al. [8] presented a computational methodology to analyze the mechanical behavior of thin composite structures with a direct application to fish skin. The proposed procedure was applied to the specific case of the fish skin structure of the *Morone saxatilis*, using a computational finite element approach. The numerical study shows that fish skin possesses a highly anisotropic response, with a softer bending stiffness in the longitudinal direction of the fish. This softer response arises from significant scale rotations during bending, which induce a stiffening of the response under large bending curvature. In Figure 3, the indentation test and results performed on the fish skin by Vernerey et al. [8] is reported. The results show that the presence of scales increases the penetration resistance of the skin by almost tenfold; moreover, scale interlocking further improves the performance of the protective system.

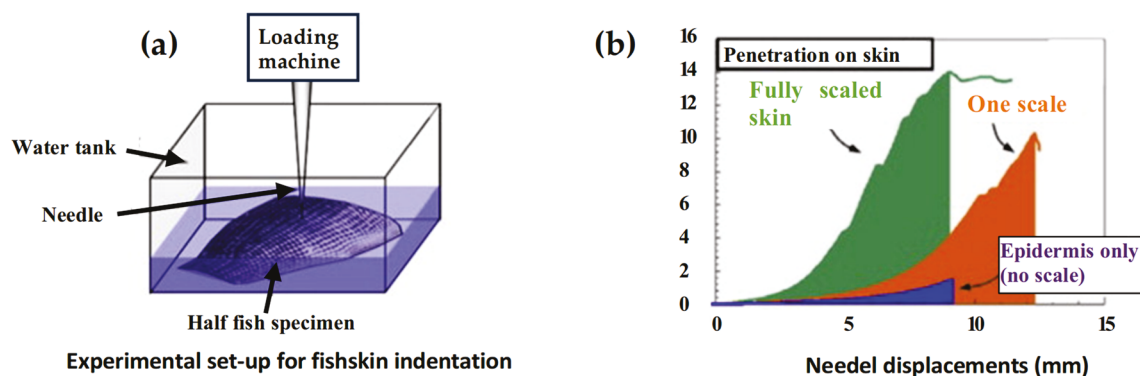


Figure 3. Illustration of the indentation test performed on fish skin and their results: (a) experimental set-up for the indentation, (b) graph of the force-deflection curve. Reproduced with permission from F. J. Vernerey et al., *Int. J. of Solids and Structures*, 51 (2014) 274–283. ©2017, Elsevier [8].

The effect of the interactions between neighboring scales is very important, as it was proved by the work of several authors (i.e., [3,4,8]). A scale resting on a softer substrate when indented by a sharp needle because of the relatively high strength of the scales and the high compliance of the substrate fails by tilting. A topologically interlocked array of scales, which is what is commonly found in nature, when subjected to puncture delays the tilting of the indented scale and can increase the puncture resistance by a factor of four. Figure 4 highlights the improvement in puncture resistance for the topologically interlocked array of scales.

Recently, Dura et al. [39,40] examined how the shape of scales affects the mechanical performance of bio-inspired protective structures. Using experiments and simulations, the researchers found that specific scale geometries optimize the balance between flexibility and stiffness [39]. In [40], Dura et al. investigated the energy absorption of 3D-printed composite structures inspired by fish scales. Low-velocity impact tests showed that the bio-inspired design significantly enhances energy absorption, making these structures suitable for impact-resistant applications. Xuechen et al. [41] explored the role of pangolin scales in pathogen defense. Using multi-omics analysis, the researchers found that the scales trap pathogens and contain antimicrobial proteins and metabolites, suggesting a complex defense system that enhances innate immunity.

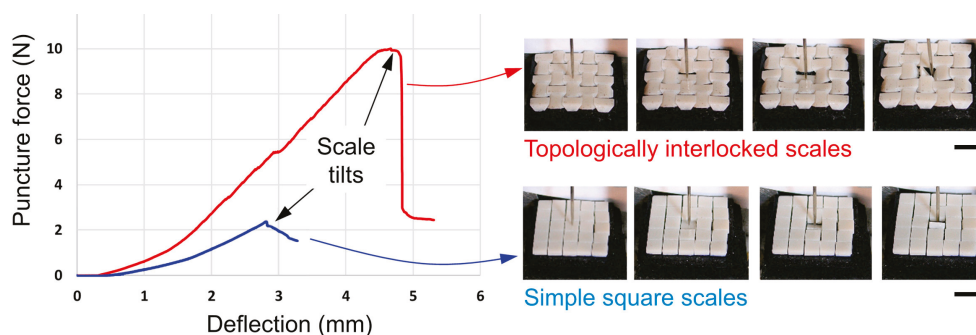


Figure 4. Puncture force–deflection curves for simple and interlocked scales with associated sequences of pictures. Both systems fail by sudden tilting of the indented scale. However, tilting is delayed in the interlocked scales, which increases the puncture resistance by a factor of four. Scale bars: 10 mm. Reproduced with permission from M. M. Porter et al., *J. of the Mech. Behavior of Biomedical Mat.*, 73 (2017) 114–126. ©2017, Elsevier [3].

Protection and flexibility are aspects that collide with each other, but the creation of protective scale structures allows for greater design possibilities to optimize both aspects as needed. For example, Martini et al. [4] in their study show that the simplified ganoid and elasmoid designs, which are close to the geometry and arrangement of the natural scales, are among the most efficient designs, providing the highest average resistance to perforation and without losing the maximum bending compliance, as compared to other designs that cover the entire surface. Natural evolution has shaped the geometry and arrangement of natural scales to maximize protective efficiency.

This study examines the puncture resistance and flexibility of natural dermal armor, offering insights into its design principles and potential applications in bio-inspired protective systems. Advances in additive manufacturing and computational modelling can boost the design, the optimization, and the production of protective structures inspired by fish scales for various applications. Here, two novel bio-inspired scale structures are presented that are based on the elasmoid designs present in fishes. Their shapes differ significantly from other artificial scales commonly described in the literature, which are typically flat. Instead, these scales feature a support that extends vertically from the substrate, transitioning into an inclined surface that serves as the protective component. Their performances are analyzed by finite element simulation. In particular, the two structures were tested both with regard to the guaranteed protection, through perforation tests, and the flexibility allowed, through flexural tests. The nonlinear flexural response is characterized by low stiffness at small curvatures due to scale sliding and significant stiffening at larger curvatures as they engage in protection. These results can guide the development of advanced, efficient protective materials inspired by natural armors.

2. Materials and Methods

2.1. Novel Scale Models

The two novel shapes proposed stand out distinctly from the flat artificial scales commonly described in the literature. These scales incorporate a vertical support extending from the substrate, transitioning into an inclined surface that functions as the protective component. The design aims to increase the distance between the top of the scale and the substrate, requiring greater deformation of the scales when subjected to pressure. This enhanced deformation allows the scales to absorb more energy, thereby improving the substrate's ability to withstand loads. In contrast, the traditional flat scale design merely provides a hard surface to shield the softer substrate and redistributes pressure over a larger area.

The spacing of the scales on the substrate has been carefully designed to allow for slight overlap at the edges, ensuring adequate protection while preserving the overall structure's lightness and flexibility. The thickness of the scales has been carefully selected to ensure they are robust enough to withstand loads by deforming without breaking, while still maintaining a lightweight profile.

2.1.1. Type 1 Bio-Inspired Scale

The first type, referred to as type 1, of bio-inspired scale is shown in Figure 5. The scale has a maximum size of 4 mm in width and 3.9 mm in length, with a height of 1.7 mm. Specifically, the scale is formed by a support base to the substrate, with 1.5 mm sides and a 0.4 mm height, and a shape similar to those seen in nature in fishes. This scale also has an inclination angle of 77° with respect to the vertical to ensure a geometric arrangement with overlapping and staggered scales.

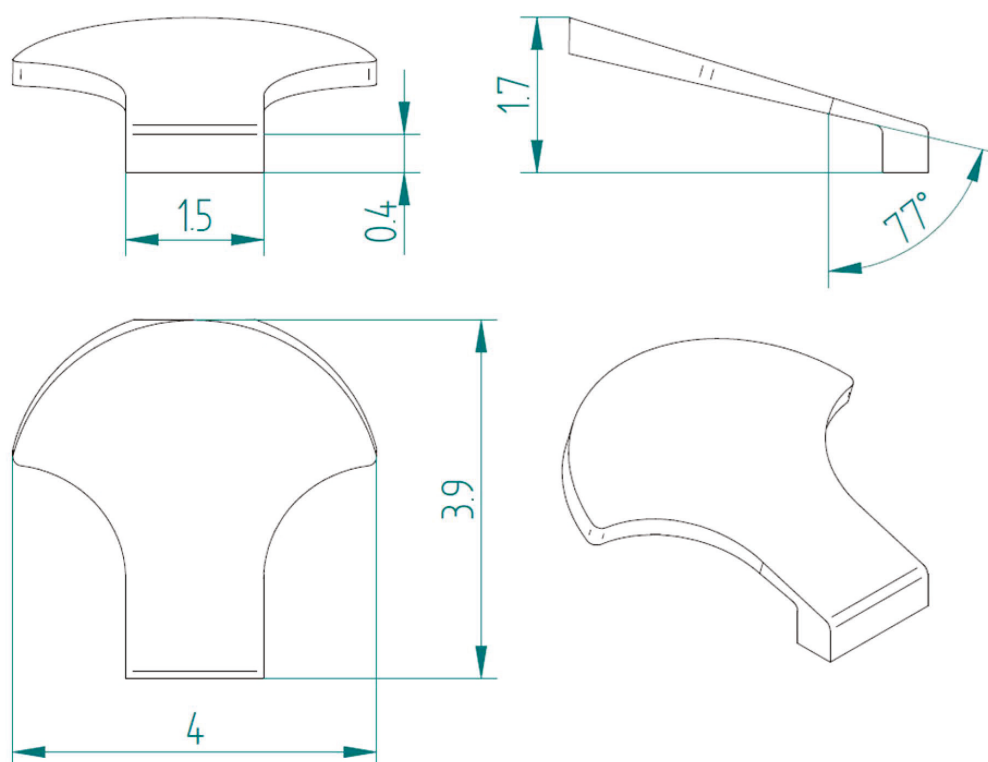


Figure 5. Type 1 bio-inspired scale with geometric dimensions in millimeters.

In nature, the scales are arranged in overlapping and staggered structures, as in Figure 6, to provide greater protection to the underlying skin. This structure is made up of three staggered columns, each of which is made up of three scales. In each column, the scales are placed at a distance of 2.6 mm from each other, while the columns are offset by 1.3 mm from each other. In this way, each scale has a distance of 1.3 mm from the next, ensuring a stronger connection than the simple overlapping scale structure. In fact, the second scale of the second column, that is, the one placed in the center of the structure, is connected to the six scales that surround it, thus allowing for greater protection, thanks to the mutual actions derived from the contacts between the various scales.

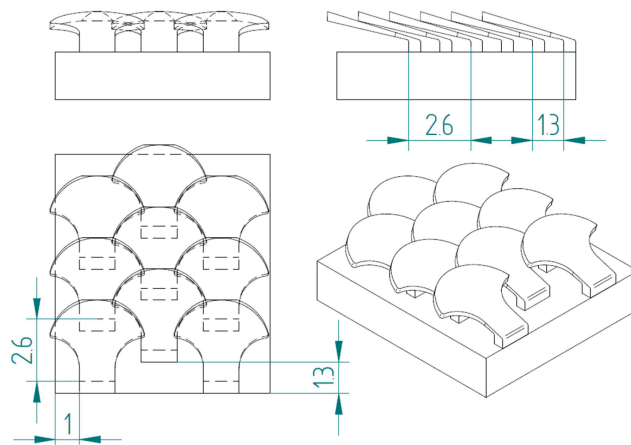


Figure 6. Type 1 bio-inspired scale structure in a configuration with overlapping and staggered scales.

2.1.2. Type 2 Bio-Inspired Scale

The second type, referred to as type 2, is shown in Figure 7.

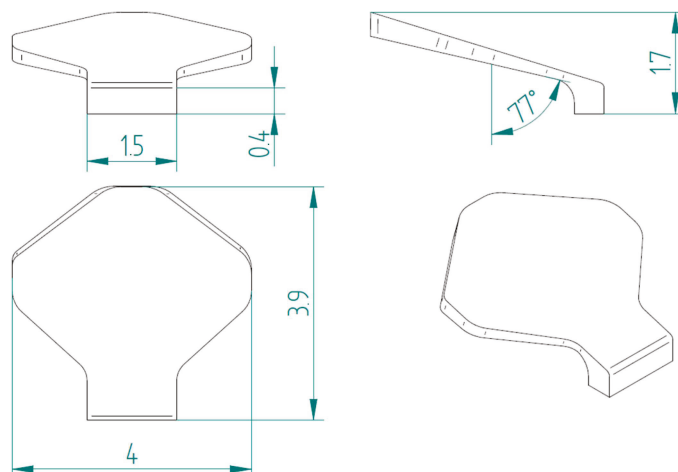


Figure 7. Type 2 bio-inspired scale with geometric dimensions in millimeters.

Figure 8 shows the structure with overlapped and staggered scales for the type 2 bio-inspired scales. The scales are spaced 2.6 mm apart, while the columns are offset by 1.3 mm.

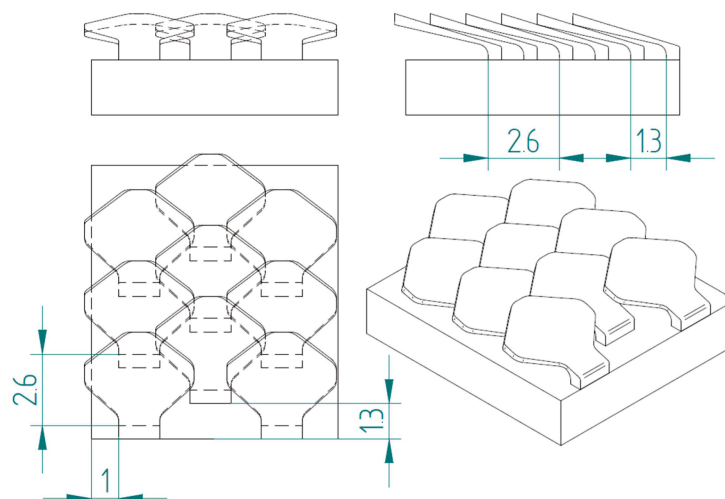


Figure 8. Type 2 bio-inspired scale structure in a configuration with overlapping and staggered scales.

2.1.3. Substrate

The substrate reproduces the skin of the fish, and it has been modeled in a prismatic form, which has a rectangular section with 9 mm and 10 mm sides, with a thickness of 2 mm, see Figure 9.

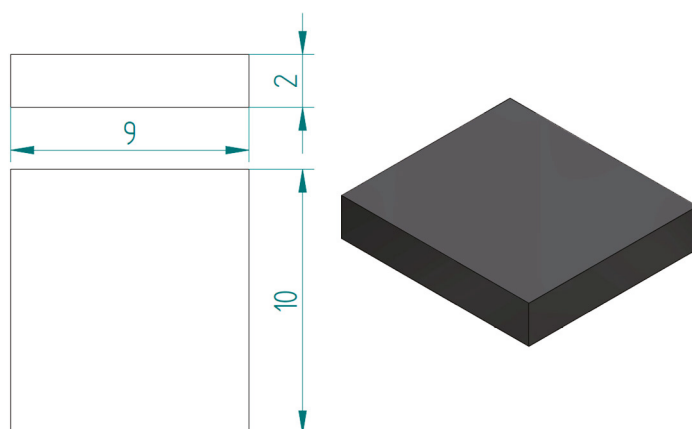


Figure 9. Substrate: rectangular 9×10 mm.

2.2. Numerical Method

Once the design of the scale structures was defined, the finite element method was used to perform numerical simulations of a puncture resistance test and a flexural strength test in order to evaluate the performance of each structural configuration with regard to the protective capacity and flexibility.

2.2.1. Material Properties

As in nature, the scales must be made of a hard material resistant to impacts and perforations, while the substrate must be made of a soft and elastic material to allow for good flexibility. It is also necessary to consider that the scales could be made via 3D printing; in this perspective, it has been assumed that the scales are made of thermoplastic polymer ABS (acronitrile butadiene styrene), while the substrate is made of polyester fabric. ABS's mechanical behavior is mainly that of a fragile material; in its stress–strain curves, the plastic behavior is almost absent. Moreover, the local stresses for the level of loads applied do not determine fracture. For this reason, for the ABS in the simulations, only the linear properties have been considered.

In particular, these properties have been considered for ABS:

- Density $\rho = 1040 \text{ kg/m}^3$;
- Young's longitudinal modulus of elasticity $E = 3 \text{ GPa} = 3000 \text{ MPa}$;
- Poisson coefficient $\nu = 0.35$.

The mechanical properties hypothesized for the polyester fabric are the following:

- Density $\rho = 1200 \text{ kg/m}^3$;
- Young's longitudinal modulus of elasticity $E = 0.5 \text{ GPa} = 500 \text{ MPa}$;
- Poisson's ratio $\nu = 0.4$.

The puncture tests require a hard tip; it was assumed to be made of structural steel, having the following properties:

- Density $\rho = 7850 \text{ kg/m}^3$;
- Young's longitudinal modulus of elasticity $E = 200 \text{ GPa} = 200,000 \text{ MPa}$;
- Poisson's ratio $\nu = 0.3$;
- Breaking strength $\sigma_r = 460 \text{ MPa}$.

2.2.2. Finite Element Model

The simulations were conducted using the commercial software ANSYS Workbench 2020. Nonlinear static analyses were performed with the large deflection option enabled and the number of substeps configured as follows: initial 200, minimum 100, and maximum 1000. Linear tetrahedral elements were utilized for the mesh, with element sizes assigned as follows: the scales were meshed with a body sizing of 0.15 mm and a face sizing of 0.10 mm. Additionally, in the type 1 bending simulations, the lateral faces that come into contact under high deformation levels were refined with a face sizing of 0.05 mm. The substrate was meshed with elements sized at 0.15 mm. The “Stabilization” option was left on the default: “Program controlled”.

Contact pairs are automatically created by the software when the CAD file is imported, but the properties are always set as “bonded” so it is a task of the user to change this setting where needed. For the scale–scale and scale substrate contact, a “frictional” type of contact was selected with a coefficient of friction equal to 0.2.

2.2.3. Puncture Resistance Test

The first test performed was puncture resistance; it was performed through the action of a hard tip, which causes piercing stress on the structure. The simulations reproduce the puncture tests performed by Martini et al. [4]. Figure 10 shows the modeled tip.

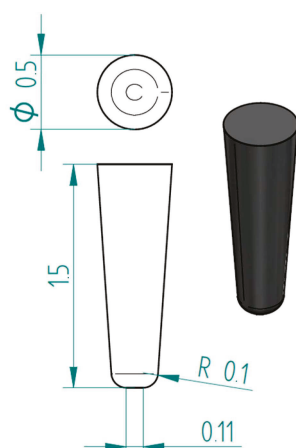


Figure 10. Pointed indenter that reproduces a steel needle. Dimensions are in millimeters.

The test is simulated by binding the lower surface of the substrate with a joint and letting the drill bit descend 1.5 mm from the initial position, which is about 0.6 mm higher than the surface of the scale. In particular, in all tests, the tip is placed at a height of 3.5 mm from the upper edge of the substrate and is made to descend along the vertical, impeding displacements other than vertical movement. For both structures, a series of comparative tests were performed to evaluate the two types of scales.

2.2.4. Flexural Test

Flexural tests were carried out on a configuration with overlapping and staggered scales, since it is believed that this is the most competitive. In the tests, since the scales on the “extrados side” do not produce any noteworthy stiffening compared to the bare polyester substrate because in this configuration the scales move away from each other, the scales were positioned on the “intrados” side of the bent samples in order to test the way in which they increase the rigidity of the sample through their interactions. The bending tests must be performed in two orthogonal directions, since the scales interact differently in the direction corresponding to the main bending direction in the animals with respect to the direction perpendicular to the latter.

To enable high bending angles without encountering convergence issues, the boundary conditions were meticulously designed. The lateral surfaces of the substrate were connected to specific points, referred to as “remote points” in ANSYS, where rotations were applied. Additional boundary conditions were introduced solely to prevent labilities while allowing the substrate to deform freely. Refer to Figure 11 for the configurations used in the flexural tests.

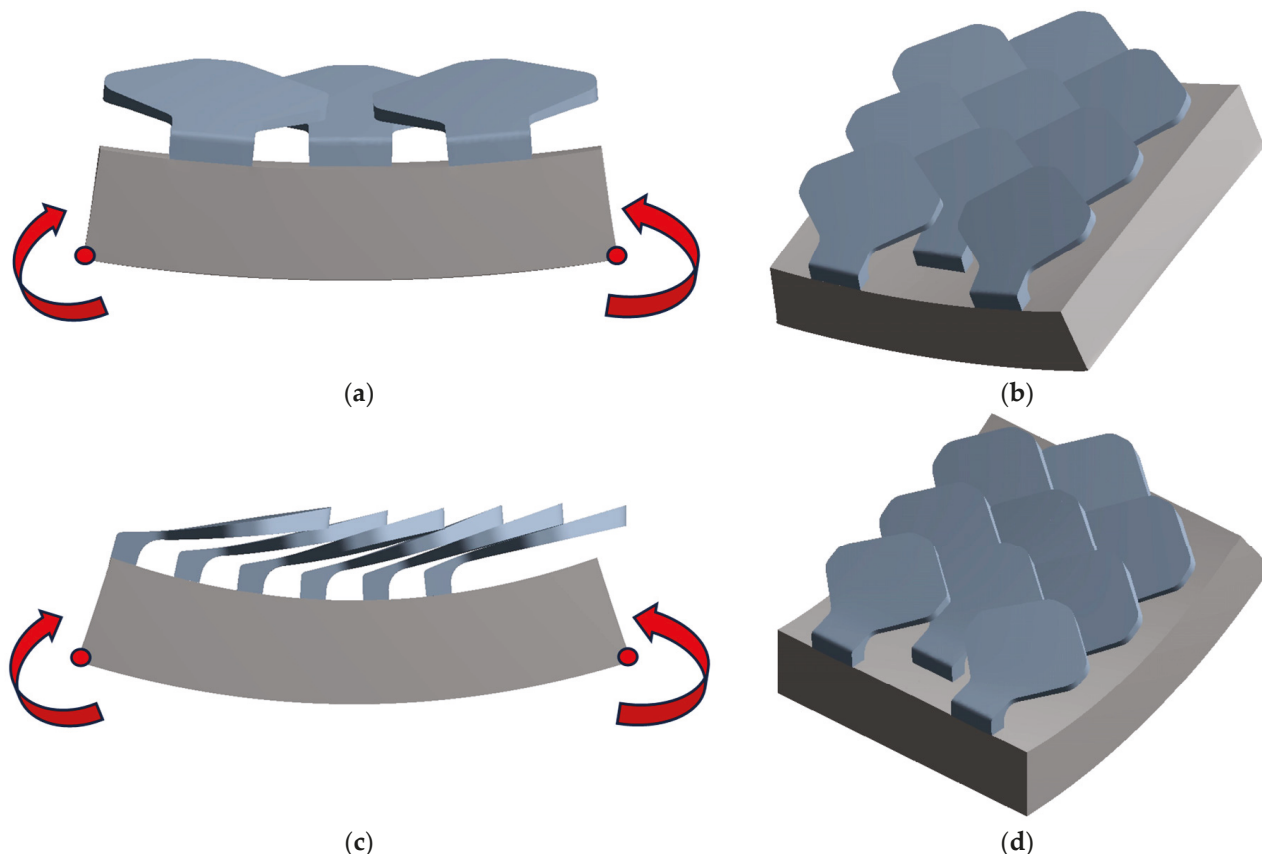


Figure 11. Flexural tests: (a) type 1—scale structure 1, (b) type 2—scale structure 1, (c) type 1—scale structure 2, (d) type 2—scale structure 2.

3. Results

3.1. Type 1 Bio-Inspired Scale

For the overlapped and staggered scale structure, three different perforation tests were performed, in which the position of the tip was changed with respect to the perforated scale.

3.1.1. Simulation Number 1: Tip at the Center of the Scale

Figure 12 shows a schematic relating to the first simulation performed. The interconnections between the various scales in the configuration with overlapping and staggered scales support the scale where the tip deforms as a result of mutual contact but help to reduce the deformation undergone by the scale subjected to perforation. In Figure 13a,b, a map of the displacements in the z direction, respectively, with and without tip, is shown. The maximum displacement in z of the scale is 0.83 mm; see Figure 13b. The critical punching force value detected is $F_p = 144$ N.

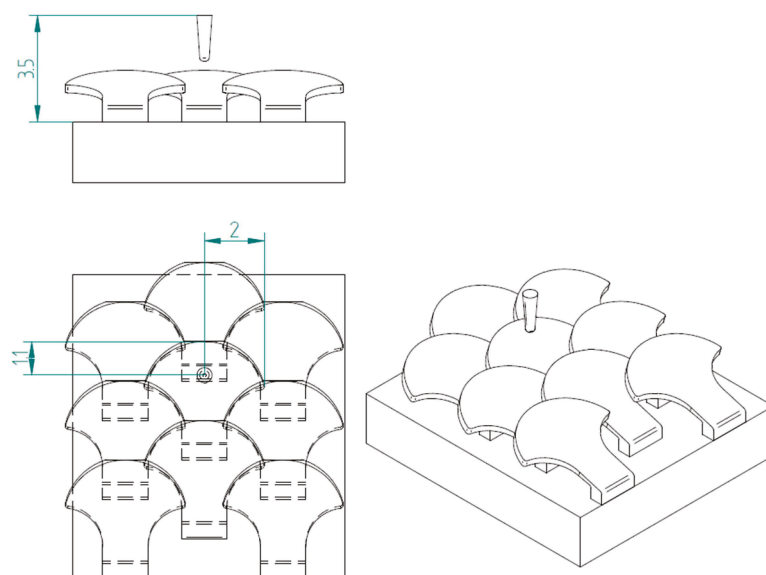


Figure 12. Schematic diagram of perforation—test n. 1—type 1 bio-inspired scale structure—overlapping and staggered configuration.

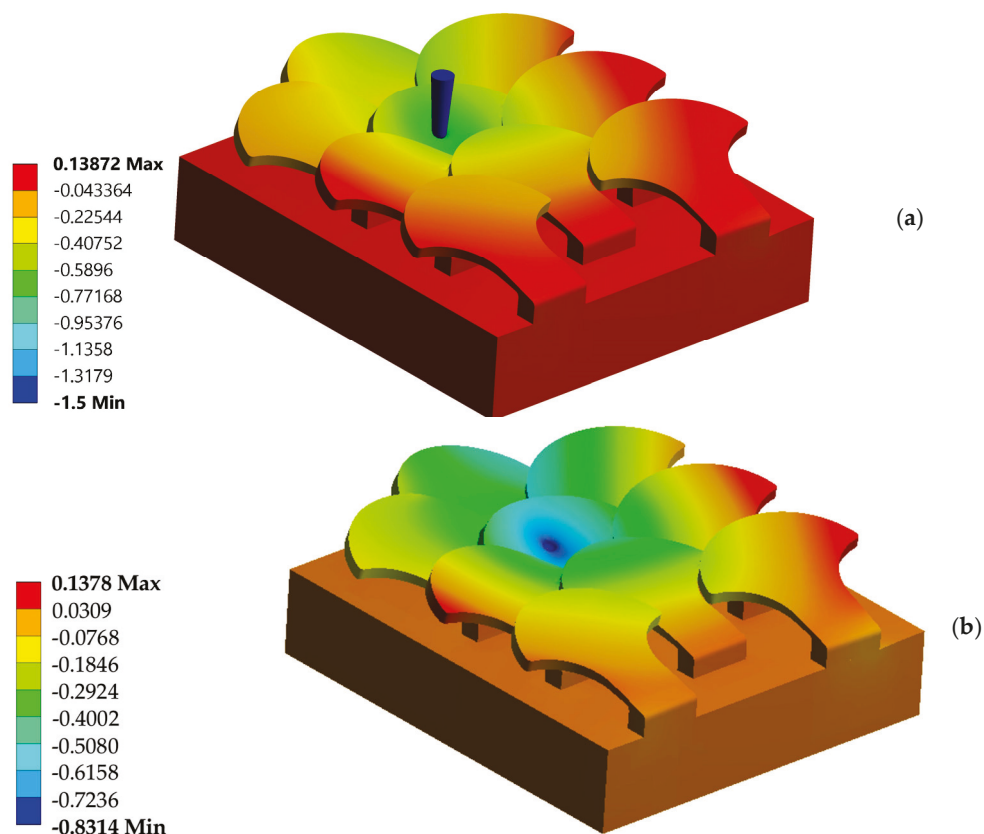


Figure 13. Map of displacements—puncture resistance test—test n. 1—type 1 bio-inspired scale structure—overlapping and staggered configuration: (a) with and (b) without the tip.

3.1.2. Simulation Number 2: Tip Near the Left Edge of the Scale

Figure 14 shows the schematization of the subsequent perforation test performed, in which the tip acts near the left edge of the previously perforated scale. In particular, the tip is positioned at a distance of 1.1 mm from the upper edge of the scale, as in the previous case, and at a distance of 0.8 mm from the left end of the scale.

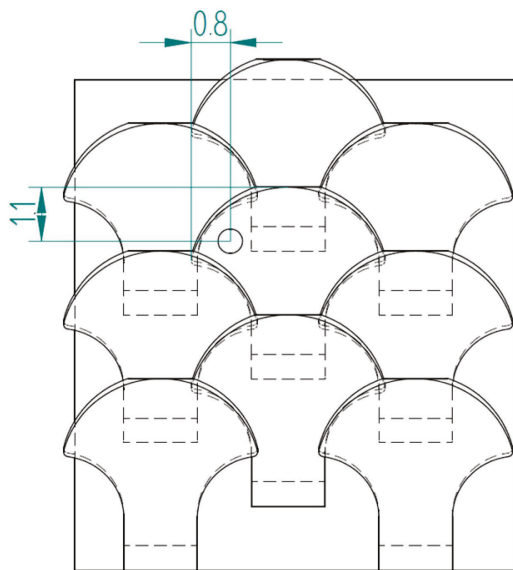


Figure 14. Schematic diagram of perforation test n. 2—structure of bio-inspired scales of type 1—configuration with overlapping and staggered scales.

Figure 15 shows that the scale tends to tilt to the left due to the action of the tip placed there. In this case, the maximum displacement in z of the scale is 0.85 mm, slightly higher than seen in the previous case. Overall, there is a greater deformation of the scale. In fact, the critical drilling force value detected is $F_p = 56$ N, much lower than the value obtained if the tip acts symmetrically with respect to the scale.

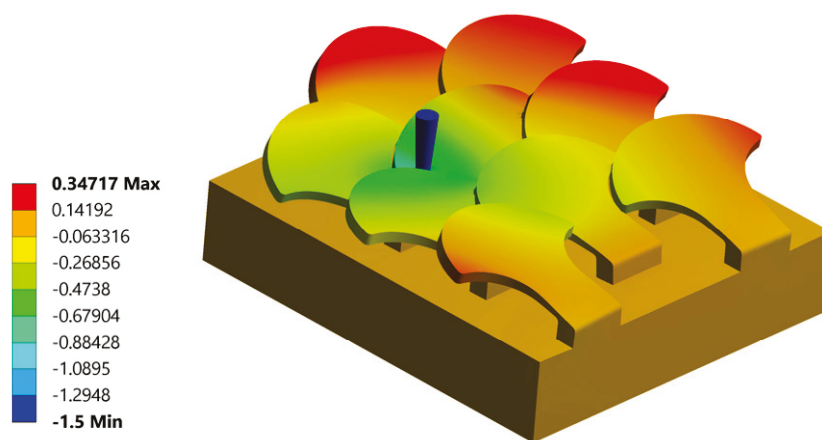


Figure 15. Perforation test n. 2 bio-inspired structure 1—overlapping and staggered scales: total displacements in mm.

3.1.3. Simulation Number 3: Tip Near the Upper Edge of the Scale

The last simulation performed is schematized in Figure 16. In the last test, the tip acts symmetrically with respect to the scale, but near the upper edge, i.e., at a distance of 0.4 mm, as shown in Figure 16.

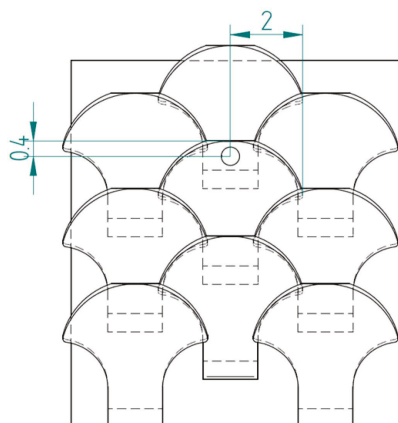


Figure 16. Schematic diagram of perforation test n. 3—type 1 bio-inspired scale structure—configuration with overlapping and staggered scales.

As shown by Figure 17, the action of the tip this time causes significant deformations towards the scale. In fact, the maximum displacement in z increases considerably compared to the previous cases, reaching 1.05 mm in the area of the tip impression. To deform the scale, however, the tip must apply greater force than test number 2, in which the tip acted near the left edge of the scale. In fact, the critical force value detected is $F_p = 136$ N, greater than the value found for simulation number 2. This is due to the fact that, in the latter case, the tip acts symmetrically with respect to the scale, favoring contacts between the nearby scales, which increase the protection of the structure. As noted, the critical drilling force of this configuration is lower than that found in test number 1, due to the fact that the tip is located closer to the edge of the scale, causing a greater bending load on it.

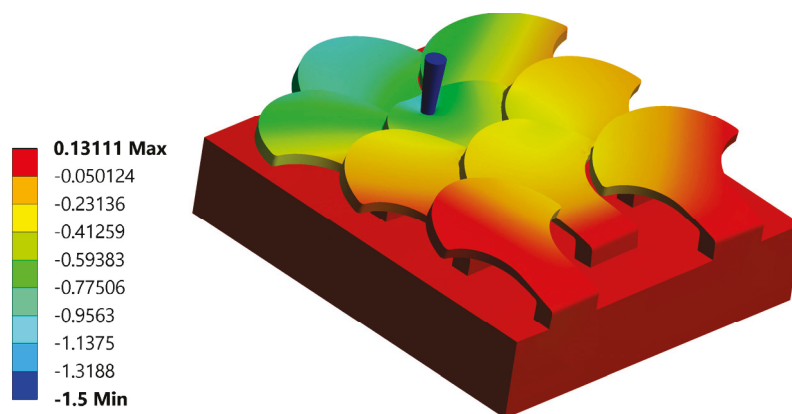


Figure 17. Perforation test n. 3 bio-inspired structure 1—overlapping and staggered scales: total displacements in mm.

3.2. Type 2 Bio-Inspired Scale Structure

The simulations performed for the type 2 bio-inspired scale structure are the same as those performed for the type 1 bio-inspired structure. These are detailed in the following paragraphs.

3.2.1. Simulation Number 1: Tip Near the Center of the Scale

The first analysis is schematized in Figure 18. The tip is placed in the same position as seen for the type 1 bio-inspired structure.

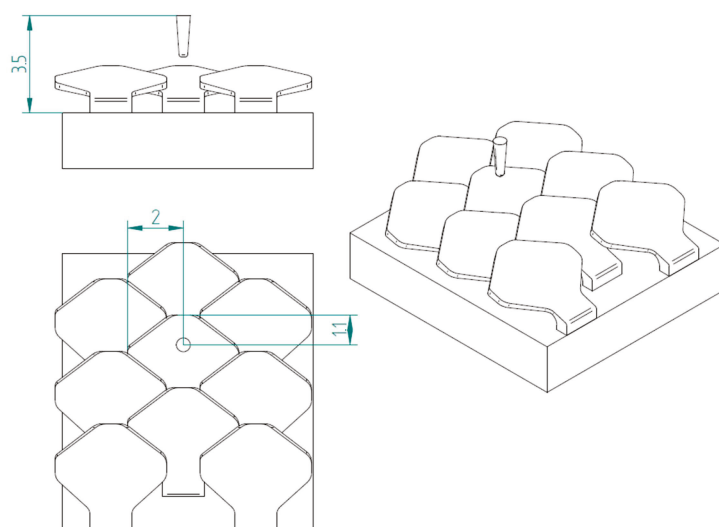


Figure 18. Schematic diagram of perforation test n. 1—type 2 bio-inspired scale structure—configuration with overlapping and staggered scales.

Analyzing the displacement in z of the single perforated scale, the same behavior seen with type 1 bio-inspired scales is recorded. In this regard, Figure 19 shows the displacement along z . The maximum displacement suffered by the scale is 0.83 mm, in accordance with the value found for the type 1 scale structure, but for this configuration, there is a reduction in the overall deformation suffered by the bio-inspired type 2 scale compared to that seen for the type 1 scale in the same load condition. This aspect is certainly linked to the geometric shape of the type 2 scale, which ensures better interconnection between the various scales. For this load condition, the critical piercing force is $F_p = 156$ N. The critical piercing force value detected for this configuration is greater than the critical piercing force evaluated for the same load configuration with the type 1 scale structure.

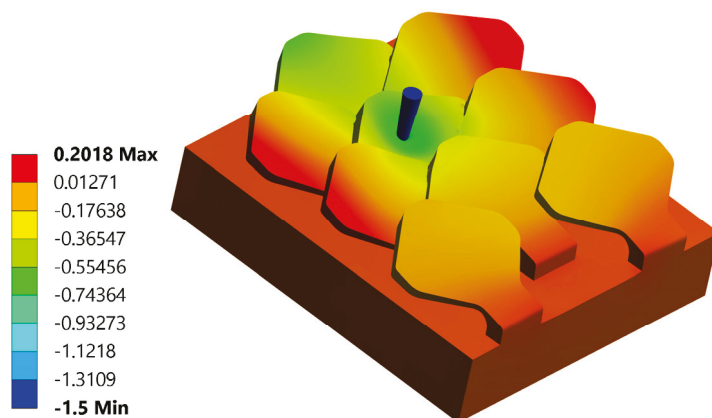


Figure 19. Perforation test n. 1 bio-inspired structure 2—overlapping and staggered scales: total displacements in mm.

3.2.2. Simulation Number 2: Tip Near the Left Edge of the Scale

In this case, the tip is placed near the left edge of the scale; see Figure 20.

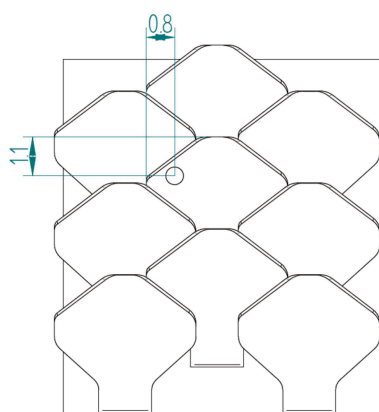


Figure 20. Schematic diagram of perforation test n. 2—type 2 bio-inspired scale structure—configuration with overlapping and staggered scales.

Figure 21 shows the final deformed configuration. This detail highlights the asymmetric deformation suffered by the scale. Furthermore, the maximum displacement value in z suffered by the scale in the area of the tip is 0.83 mm. The latter is equal to the displacement in z suffered by the scale during simulation number 1, in which the tip acted near the center of the scale. This result shows that the structure with overlapping and staggered scales with type 2 bio-inspired scales is very efficient even in the presence of a perforating load that acts asymmetrically against the scale. The improved efficiency of the bio-inspired type 2 scale is highlighted once again by the detected critical piercing force, which is equal to $F_p = 82$ N. This value is much higher than that found for the type 1 bio-inspired scale structure with the same load conditions.

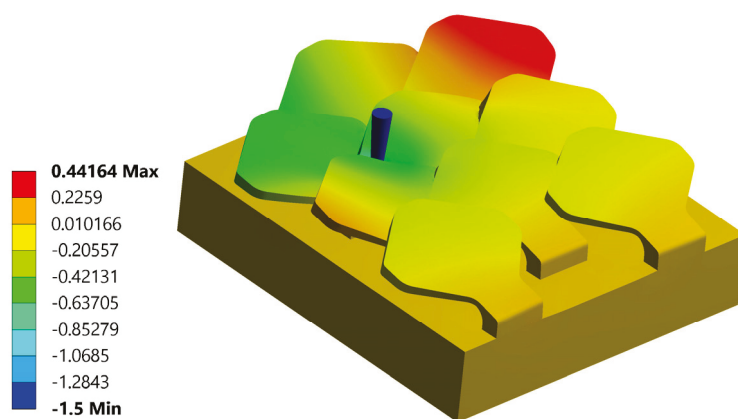


Figure 21. Perforation test n. 2 bio-inspired structure 2—overlapping and staggered scales: total displacements in mm.

3.2.3. Simulation Number 3: Tip Near the Upper Edge of the Scale

The last perforation test performed is schematized in Figure 22. Below, in Figure 23, the map of the total displacements is illustrated. In this load condition, the tip causes significant deflections to the affected scale, thus generating significant deformations also to the neighboring scales. From the images below, you can appreciate the symmetrical behavior of the structure following the application of the load.

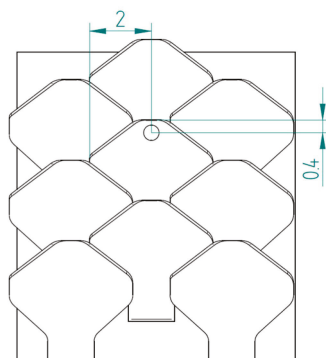


Figure 22. Schematic diagram of perforation test n. 3—type 2 bio-inspired scale structure—configuration with overlapping and staggered scales.

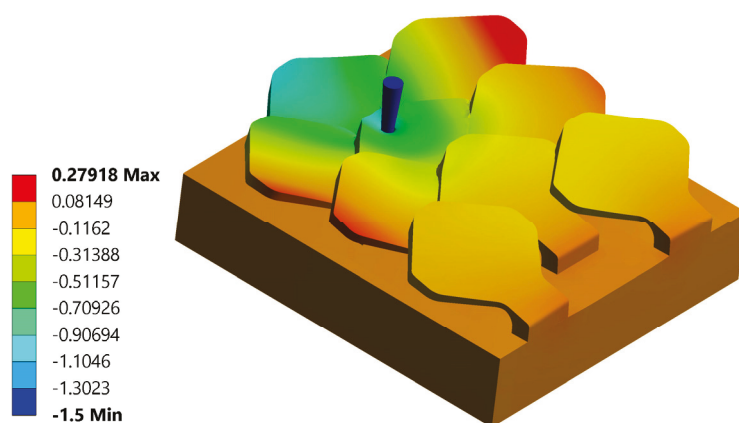


Figure 23. Perforation test n. 3 bio-inspired structure 2—overlapping and staggered scales: total displacements in mm.

This best illustrates the significant deflections suffered by the scale. In fact, the maximum displacement detected in the load application area is 1.03 mm. This value is comparable with the outcomes of simulation n. 3 performed for the type 1 structures. The interconnections between the neighboring scales, however, cause the structure to offer higher resistance to these deflections. In fact, the critical drilling force value is $F_p = 158$ N, the highest recorded in all the eight tests performed for overlapping and staggered scale structures.

3.3. Comparison Between the Two Bio-Inspired Scales

It emerged that the bio-inspired type 2 scale is more resistant to perforation than the bio-inspired type 1 scale.

To have a quantitative comparison between the two types of scales, we decided to use the perforation response surfaces as the point of action of the perforating tip changes, similarly to what was carried out by R. Martini, Y. Balit, and F. Barthelat [4].

To do this, it was necessary to perform six additional perforation tests compared to the three tests previously performed for the overlapped and staggered structures with type 1 and type 2 scales.

Figure 24 shows the schematizations of the six additional tests performed for a type 1 bio-inspired scale structure.

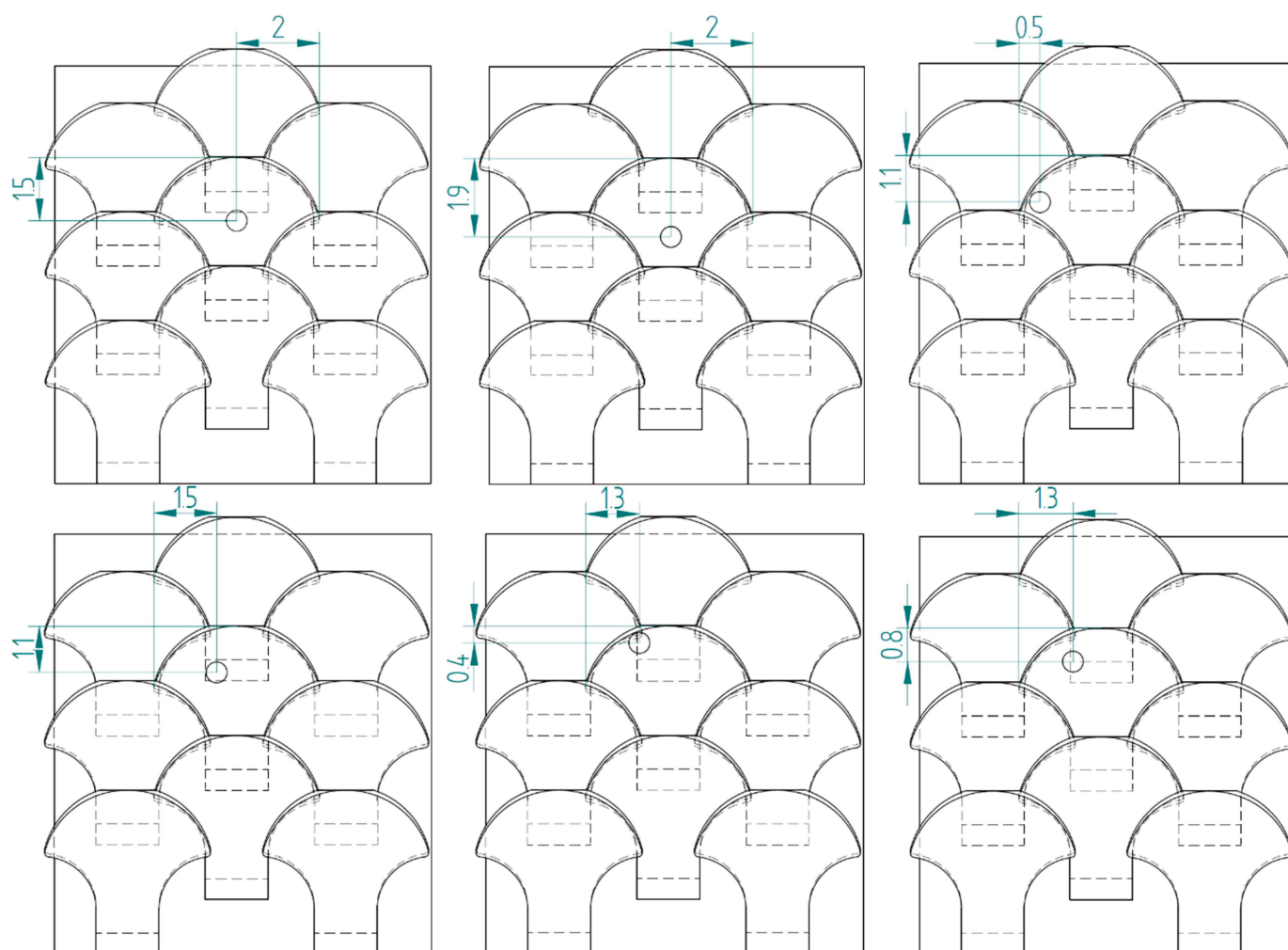


Figure 24. Schematization of six additional perforation tests—type 1 bio-inspired scale structure—overlapping and staggered scale configuration.

Figure 25, on the other hand, shows the schematization of the six additional tests carried out for the bio-inspired type 2 scale structure. The tests performed are the same as those carried out for the type 1 scale structure, in order to carry out a direct comparison among the data found.

In the various analyses performed, the tip varies with respect to the axis of symmetry of the perforated scale, with any offsets only towards the left part of the scale. This is carried out because the same results are obtained if the tip is positioned specularly with respect to the axis of symmetry of the scale. Below, in Table 1, the critical penetrating force values obtained in the various analyses performed for the structure with bio-inspired stairs of type 1 and type 2 are shown. Table 1 reports both the results of the new group of analyses 4, 5, 6, 7, 8, and 9 and the results of analyses 1, 2, and 3 previously illustrated in the appropriate sections.

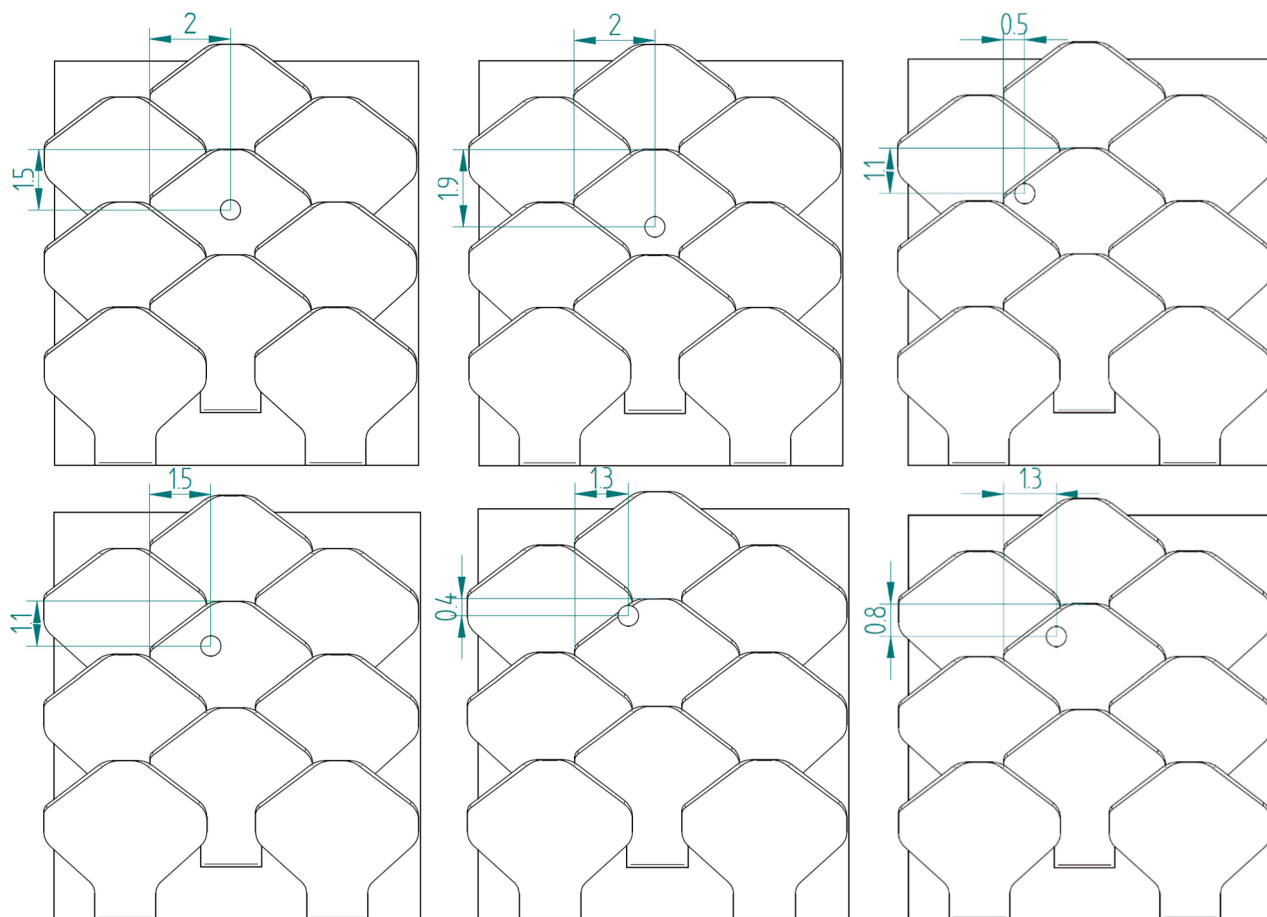


Figure 25. Schematization of six additional perforation tests—type 2 bio-inspired scale structure—overlapping and staggered scale configuration.

Table 1. Values of critical drilling force F_p with variation in the penetrator action point.

Number of Analyses	Point of Action of the Tip with Respect to the Center of the Scale		Critical Drilling Force F_p (N)	
	Offset X (mm)	Offset Y (mm)	Structure Bio 1	Structure Bio 2
1	0	0.85	144 N	156 N
2	−1.2	0.85	56 N	82 N
3	0	1.55	136 N	158 N
4	0	0.45	60 N	70 N
5	0	0.05	16 N	23 N
6	−1.5	0.85	39 N	70 N
7	−0.5	0.85	129 N	142 N
8	−0.7	1.55	105 N	131 N
9	−0.7	1.15	134 N	130 N

The position where the tip strikes the scale is also included in the table. This position is evaluated with offsets in the x and y directions with respect to the center of the scale, as shown in Figure 26.

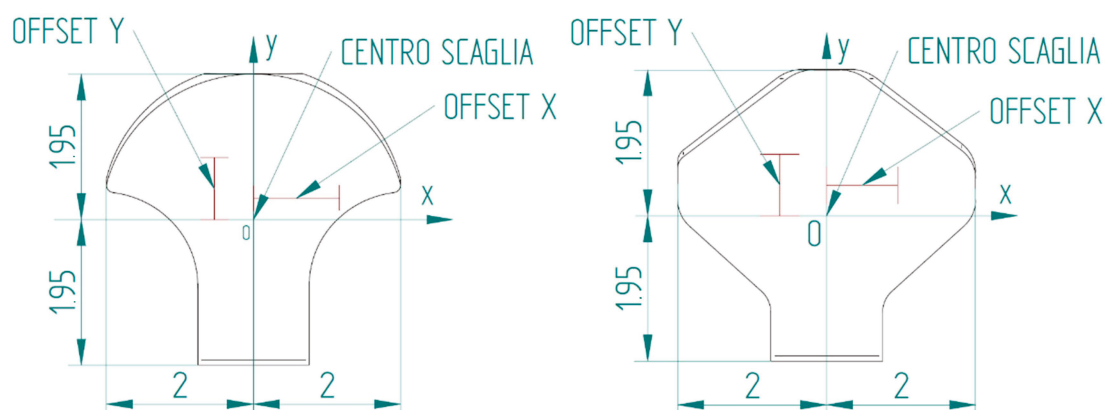


Figure 26. x-y coordinate system used to report the position where the tip hits the scale. Type 1 bio-inspired scale on the left; type 2 bio-inspired scale on the right.

Table 1 allows for two interesting considerations:

- Very low puncture resistance values were found in analysis number 5, in which the tip is placed near the center of the scale. This is due to the fact that at that point, the affected scale is more exposed, since it is less helped by the nearby scales, due to the degree of overlap used;
- Analysis number 9 is the only one in which the type 1 bio-inspired scale structure has a greater perforation resistance than the type 2 bio-inspired scale structure.

Having performed nine different perforation simulations, it is possible to reconstruct the trend of the critical perforation force as the point where the scale is hit. In Figure 27a,b, the trend of the critical drilling force as the offset changes in x and y is shown with respect to the center of the scale for a type 1 bio-inspired scale structure.

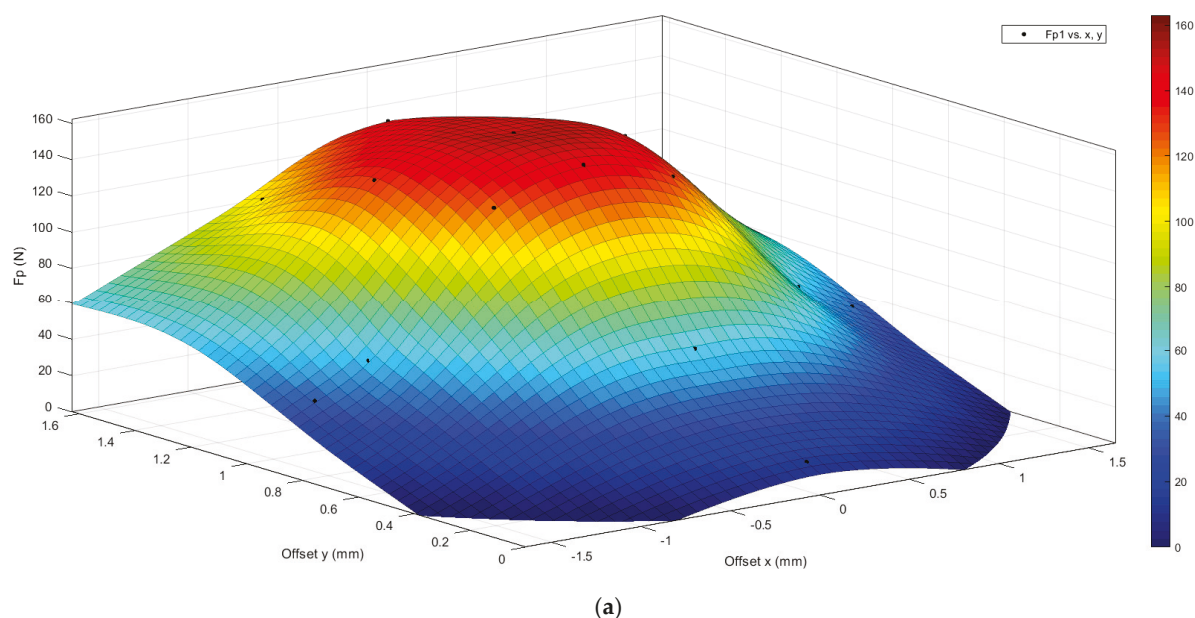


Figure 27. Cont.

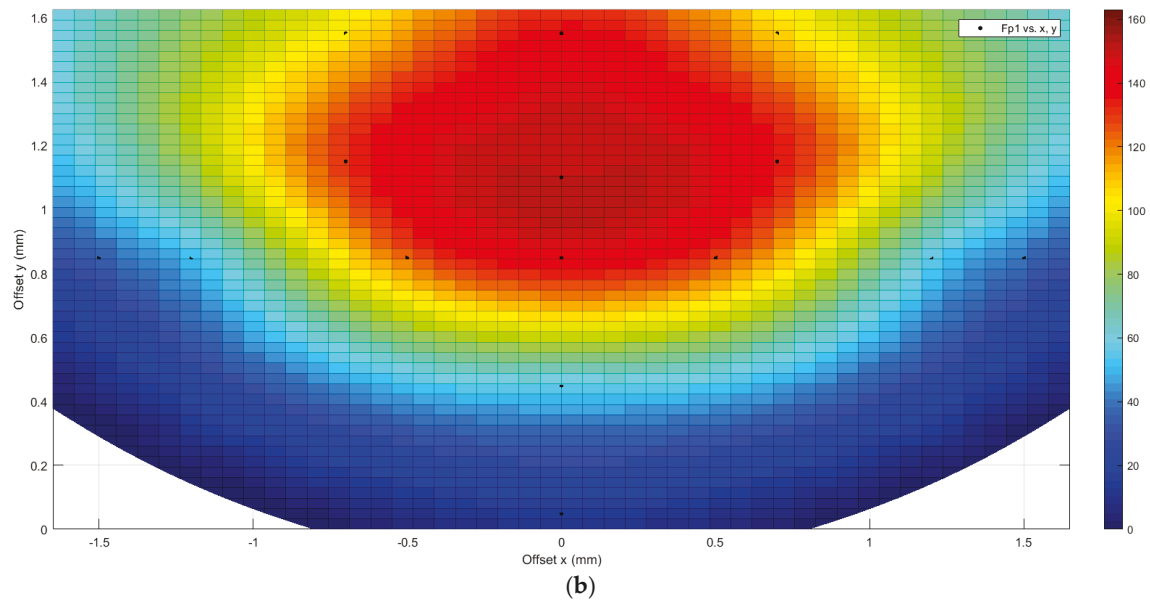


Figure 27. Map of critical drilling force as the action point of the drill bit changes (offset in x and in y)—type 1 bio-inspired scale structure—high response surface, low grid of force values in x and y (the colored bar indicates the entity of the punching force detected: dark red, max values; blue, minimum values).

Figure 28 shows the same graphs for a type 2 bio-inspirate scale structure.

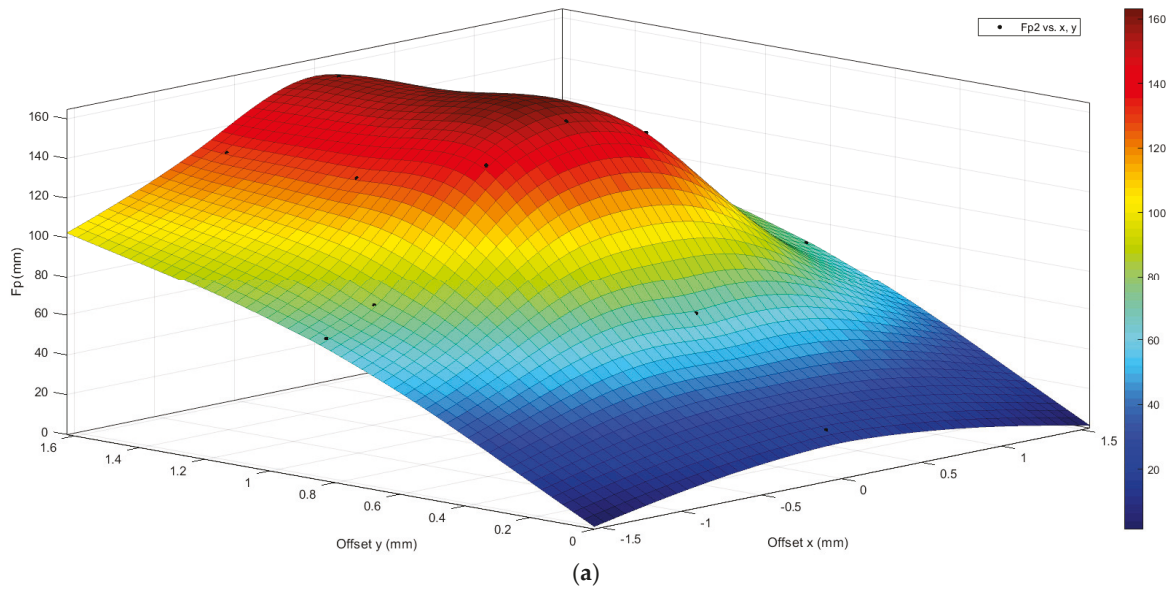


Figure 28. Cont.

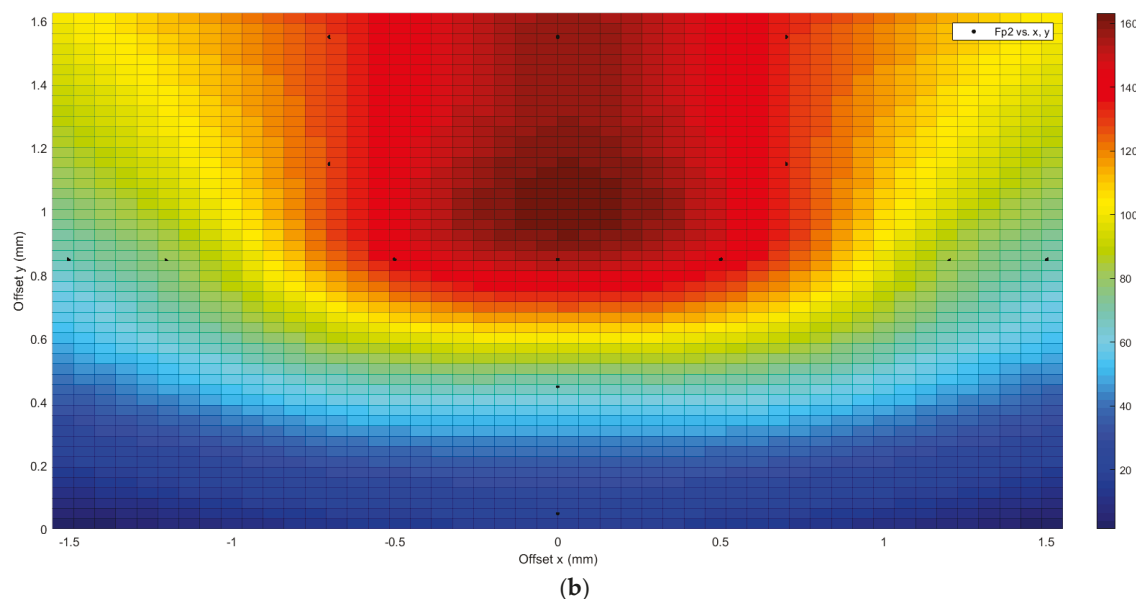


Figure 28. Map of critical drilling force as the action point of the drill bit changes (offset in x and in y)—type 2 bio-inspired scale structure—high response surface, low grid of strength values in x and y (the legend indicates the entity of the punching force detected: dark red, max values; blue, minimum values).

The graphs shown are constructed by interpolation starting from the results obtained in the nine analyses shown in Table 1. The graphs once again highlight the greater puncture resistance of the type 2 bio-inspired scale structure compared to type 1.

Figure 29 presents an example of the force as a function of the tip displacement for the case where the highest reaction force was recorded, specifically during perforation test n. 1 of bio-inspired structure 2.

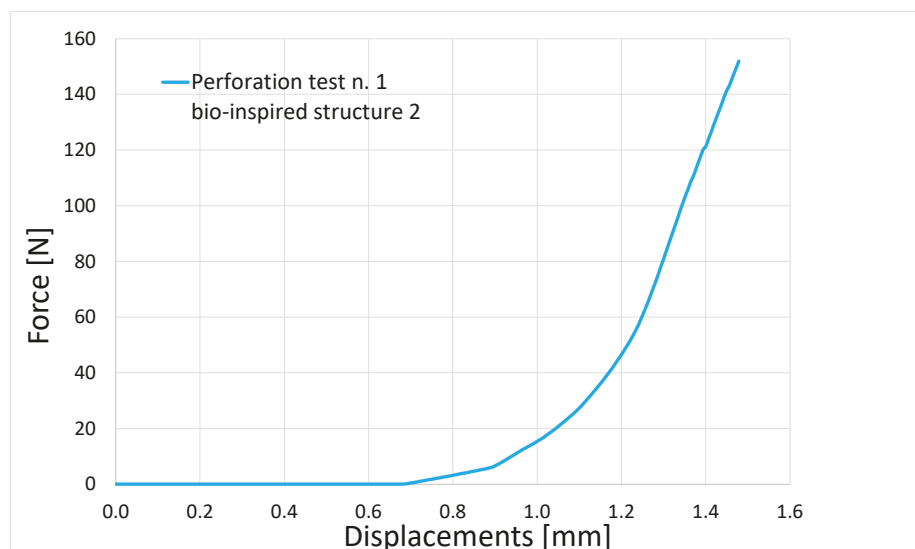


Figure 29. Force as a function of the tip displacement for the case where the highest reaction force was recorded: perforation test n. 1 of bio-inspired structure 2.

3.4. Flexural Test

3.4.1. Type 1 Bio-Inspired Scale Structure

Figure 30a,b show the final deformed configuration for bending test n. 1 for the type 1 bio-inspired scale structure. Once again, it is necessary to define a parameter that

characterizes the performance of the structure following the load applied. As described in Section 2.2.4, the lateral surfaces of the substrate were connected to specific points, referred to as “remote points” in ANSYS, where rotations were applied to influence the connected surfaces. Additional boundary conditions were introduced solely to prevent instability while allowing the substrate to deform freely. Both lateral surfaces were rotated by 40 degrees. In Flexural Test 1, the total bending moment, calculated as the sum of the moments at the two distinct remote points, is $M_1 = 1023 \text{ Nmm}$.

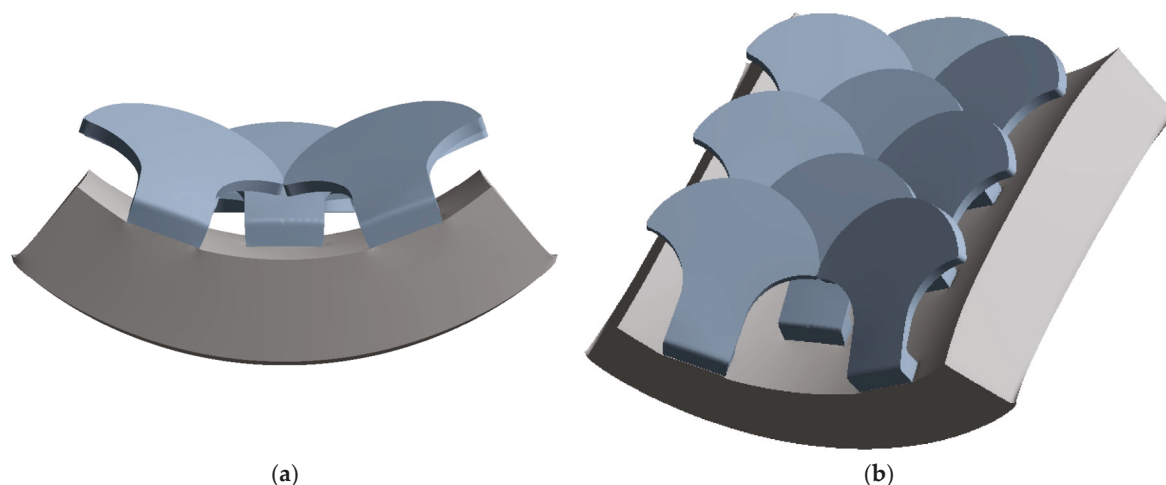


Figure 30. Flexural test—type 1—bio-inspired structure 1.

The second bending test is illustrated in Figure 31 below, which shows the final deformed configuration at the end of the analysis. The bending of the substrate is partially prevented by the scales, which come into contact with the substrate itself. The bending moment detected for the second analysis is $M_2 = 1505 \text{ Nmm}$.

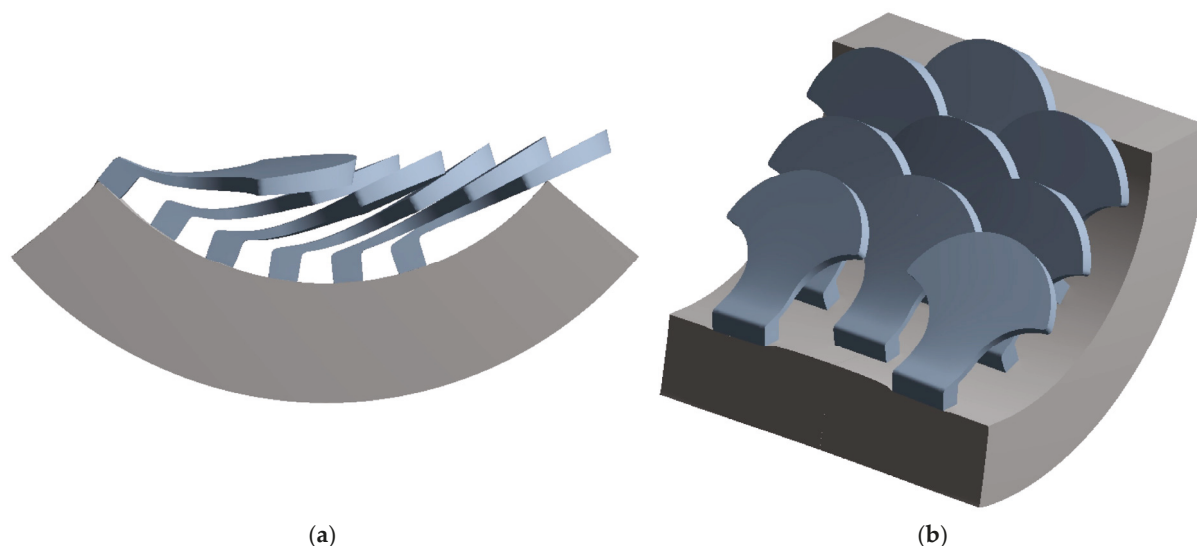


Figure 31. Flexural test—type 2—bio-inspired structure 1.

3.4.2. Type 2 Bio-Inspired Scale Structure

The same tests seen in the previous paragraph are repeated for the type 2 bio-inspired scale structure. Figure 32 shows the total deformed configuration from bending test n. 1.

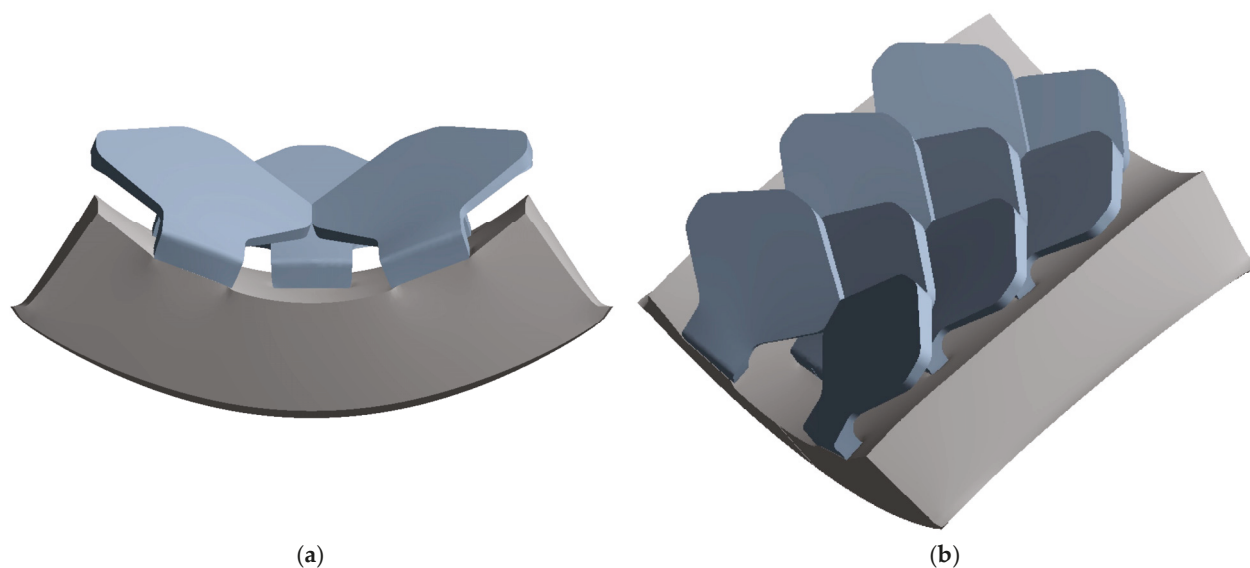


Figure 32. Flexural test—type 1—bio-inspired structure 2: (a) Front view, (b) Isometric view.

The measured bending moment is $M_1 = 1092$ Nmm. This value exceeded the value of 1023 Nmm observed in the bending analysis of the test conducted for the type 1 scale structure.

Finally, Figure 33 shows the deformed structure from bending test number 2.

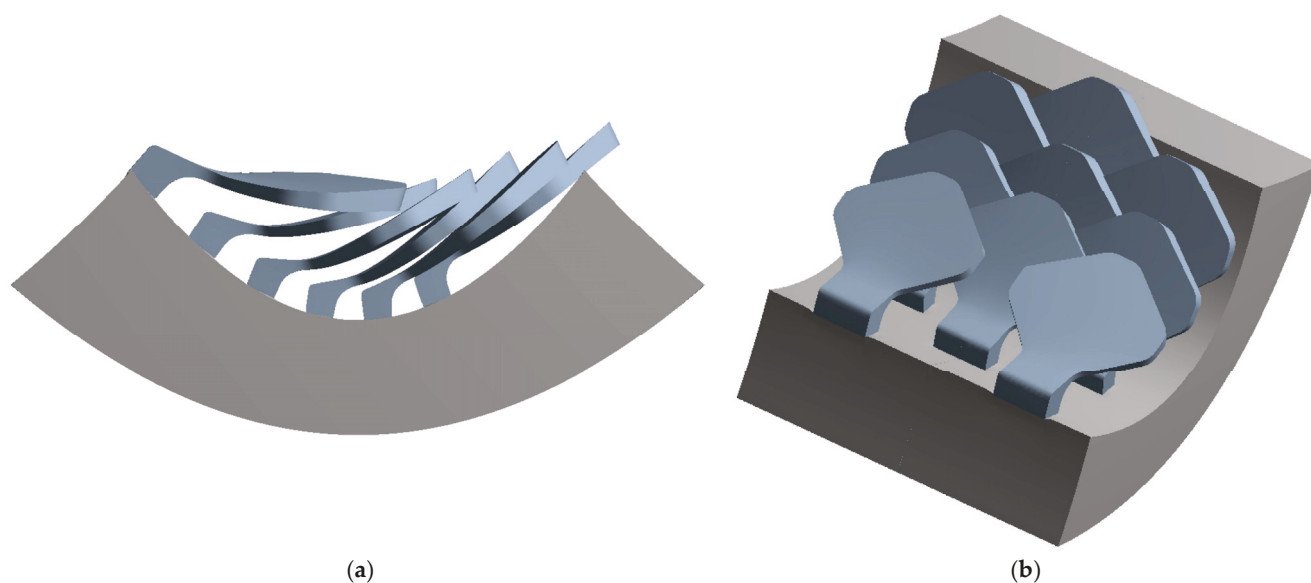


Figure 33. Flexural test—type 2—bio-inspired structure 2: (a) Front view, (b) Isometric view.

The bending moment detected is $M_2 = 1574$ Nmm, greater than the value of 1505 Nmm found for bending analysis number 2 performed for the type 1 scale structure.

4. Discussion

A small variation in the geometry of the scale can greatly influence the behavior of the structure in terms of puncture resistance and flexibility. This can be seen in the comparison between the type 1 bio-inspired scale and the type 2 scale. These two scales, the same in material, are similar geometrically: they differ from each other in small features, while maintaining the same overall dimensions. However, we have found that the bio-inspired

type 2 scale is more efficient in terms of puncture resistance than the bio-inspired type 1 scale, while the latter guarantees slightly better flexural behavior.

In puncture resistance tests, the type 2 scale outperforms the type 1 scale primarily due to its shape, which results in a greater degree of overlap with the surrounding scales, and partially from its shape. Since the distribution of the scales on the substrate is identical for both types, it does not influence the comparison.

In the flexural test for type 1, the sliding of the two outer rows of scales over the central row initially generated similar friction forces in both cases, as the differing shapes of the scales had minimal impact on friction, until higher bending angles were reached. At these higher angles, the outer rows of scales come into contact along their thickness. Due to the distinct shapes of the two scale types, the forces exchanged during this contact differ.

For the flexural test of type 2, at high bending angles, all the scales undergo significant bending. Since the bending stiffness varies between the two scale types due to their unique shapes, the results diverge.

Furthermore, the performance of the scale structure is strongly influenced by the distance between the various scales, as well as by their arrangement. The closer they are to each other, the greater the puncture resistance, but at the same time, flexibility is reduced and weight increases.

Depending on the intended application of the protective system, you can choose a design that prioritizes greater puncture resistance or one that is lighter and more flexible.

5. Conclusions

The purpose of this work was to continue studies on the creation of protective structures inspired by fish scales. These can be designed for various purposes, such as bullet-proof vests, protective gloves, fireproof systems, or others.

Two bio-inspired protective systems were designed and tested. Specifically, two types of scales were modeled using 3D CAD software SolidEdge 2020. Then, a configuration with overlapping and staggered scales was defined.

The proposed novel scale design differs from traditional flat artificial scales by incorporating a vertical support that transitions into an inclined protective surface. The design aims to increase the distance between the top of the scale and the substrate, requiring greater deformation of the scales when subjected to pressure. This enhanced deformation allows the scales to absorb more energy, thereby improving the substrate's ability to withstand loads. In contrast, the traditional flat scale design merely provides a hard surface to shield the softer substrate and redistributes pressure over a larger area. The scales are meticulously spaced to enable slight overlapping at the edges, offering effective protection while maintaining the structure's lightness and flexibility. Their thickness is thoughtfully chosen to provide the strength necessary to withstand loads through deformation, without compromising their lightweight nature.

The two bio-inspired structures were analyzed by finite element performance tests. Specifically, the structures were tested for their guaranteed protection using perforation tests and for their flexibility using flexural tests. The execution of these simulations allowed for a complete comparative analysis between the two types of scales. The analyses carried out showed that the type 2 scale is more efficient in terms of puncture resistance, while the type 1 scale has better performance in terms of flexibility.

The small differences in the two scales demonstrate that small geometric variations can significantly influence the behavior of the protective system, leaning towards greater protection or towards greater flexibility. These two aspects, in fact, influence each other and, depending on the use to be made of the protective system in question, you can opt for

a solution that guarantees greater protection at the expense of flexibility or greater capacity of movement at the expense of the guaranteed protection.

The models presented and analyzed can be considered as a starting point for subsequent analyses or modeling of structures that have the purpose of imitating the protective systems present in nature. To improve and optimize the protective systems, you can act in different ways, varying the following:

- Scale material;
- Substrate construction material;
- Geometric structure of the scale: dimensions, shape, and thickness;
- Distance between the various scales placed above the substrate;
- Types of reciprocal dispositions of the scales: with scales placed side by side, with overlapping scales, and with overlapping and staggered scales;
- The ratio between the surface area of the scale and the distance between their centers;
- The ratio between the area of the connection to the substrate and the area of the scale.

In the future, we can envision developing protective systems entirely inspired by nature, striving, if possible, to surpass the capabilities currently found in natural designs.

Future Work

Most studies on impact and penetration issues involving bio-inspired protective scales focus primarily on the behavior of individual scales. However, in many scenarios, multiple scales are subjected to extreme loading simultaneously. Future research should explore this context, particularly the complex interactions between scales under such conditions, offering a fascinating and largely uncharted avenue for investigation.

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Review

Advancements and Challenges in Tissue-Engineered Heart Valves: Integrating Biomechanics, Biomaterials, and Biomimetic Design for Functional Maturity

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Abstract

Valvular heart disease remains a major global health burden, with currently available prosthetic heart valves failing to fully reproduce the adaptive, regenerative, and long-term functional properties of native valves. Tissue-engineered heart valves (TEHVs) have emerged as a promising alternative, aiming to develop living valve replacements capable of growth, remodeling, and physiological integration. However, despite substantial progress, the clinical translation of TEHVs remains limited, indicating the need for design strategies that go beyond material selection toward functionally mature constructs. This review presents recent advances in TEHV development from a biomimetic perspective, using native heart valves as a biological reference characterized by hierarchical structure, anisotropic mechanical behavior, mechanoresponsive cell populations, immune regulation, and temporally coordinated remodeling. We integrate current understanding of valve biology and mechanobiology with advances in scaffold materials and architecture, bioactive functionalization, biomechanical conditioning, and emerging fabrication and monitoring technologies. We discuss how biomimetic scaffold designs aim to replicate native extracellular matrix organization and nonlinear mechanics, how biological cues are used to regulate thrombosis, immune response, and cell recruitment, and how dynamic bioreactor systems support functional tissue maturation through controlled mechanical stimulation. Finally, key challenges for clinical translation are highlighted, and future directions are outlined, emphasizing integrated and biomimetically informed design approaches. Overall, this review aims to define guiding principles that may accelerate the development of durable, regenerative, and clinically translatable tissue-engineered heart valves. We argue that successful TEHV translation requires synchronized control of scaffold anisotropy, immune modulation, and mechanical conditioning rather than incremental material optimization.

Keywords: tissue-engineered heart valves; biomimetic design; heart valve mechanobiology; scaffold architecture; biological cues; bioreactors; functional maturation

1. Introduction

Valvular heart disease (VHD) represents a growing global health challenge, driven particularly by aging populations, lifestyle-conditions and pathologies such as diabetes or hypertension [1–3]. According to population-based studies, the overall prevalence of moderate to severe VHD in industrialized countries is about 2.5% of the general adult population, rising sharply with age [1,4]. The clinical burden is multifaceted: VHD contributes

not only to high mortality but also to heart failure, arrhythmias, reductions in quality of life and significant healthcare costs [1–5].

To treat severe VHD, especially when the disease is symptomatic or causes significant functional impairment, valve replacement is the standard of care [3,6]. Two principal types of prosthetic heart valves are commonly used: mechanical and bioprosthetic valves. Mechanical valves are currently available in a variety of shapes, sizes and materials, and are the gold standard treatment for patients up to approximately 70 years old. They have the advantage of durability with an average life span of over 20 years [6]. However, regardless of the type of mechanical valve implanted, they necessitate lifelong anticoagulation therapy and thus impose an increased risk of bleeding or strokes, as well as lifestyle limitations for patients [3–7]. To reduce the thromboembolic complications of the mechanical valves, bioprosthetic valves, based on xenograft, allograft or homograft, have been introduced. Bioprosthetic valves' structure and geometry resemble the native valve more closely than the mechanical ones, resulting in better hemodynamic and reduced thrombus formation, avoiding the need for permanent anticoagulation therapy. However, the use of xenogenic or allogenic materials increases the risk of immunogenic reactions and disease transmission. In addition, their durability is substantially lower: structural valve deterioration (SVD), calcification, leaflet tearing or degeneration often leads to valve dysfunction and need for reoperation, especially in young patients [3–7].

Conventional prosthetic valves, whether mechanical or bioprosthetic, fail to reproduce the complex functional, mechanical, biological, and adaptive characteristics of native heart valves. In addition, these types of valve replacement lack growth, repair, and remodeling potential. An ideal heart valve substitute should therefore not only restore unidirectional blood flow, but also adapt to dynamic hemodynamic loads, support endothelialization and extracellular matrix (ECM) turnover, minimize thrombogenicity, resist calcification and mechanical fatigue, and exhibit long-term biocompatibility and immunological tolerance. These requirements are particularly critical in pediatric patients, where the inability of current prostheses to accommodate somatic growth often necessitates repeated surgical interventions [2,8,9].

Considering these limitations, tissue engineered heart valves (TEHVs) have emerged as a promising alternative. TEHVs are designed to replicate the architecture and function of natural heart valves through various tissue engineering (TE) approaches (in vitro, in vivo and in situ), utilizing living cells and biomaterials to generate constructs with regenerative properties. Heart valve tissue engineering employs a wide range of biomaterials and biofabrication strategies such as molding, electrospinning, melt electrowriting and 3D bioprinting to create either cellular or cell-free bioresorbable constructs that can ultimately yield living functional valves in vivo [3].

These approaches differ in complexity, level of biological control, and translational readiness. The in vitro TE paradigm, originally defined by Langer and Vacanti in 1993, is based on scaffold seeding with autologous cells, in vitro tissue maturation within a bioreactor and subsequent in vivo remodeling following implantation [3,10]. Key processes include cell proliferation and migration, ECM production and organization and scaffold degradation, all of which must be tightly balanced to achieve appropriate tissue maturation over time [6].

The in vivo TE approach instead exploits the human body as a bioreactor relying on tissue encapsulation of a nondegradable scaffold following subcutaneous implantation to generate a collagen-rich matrix that can later be harvested and used as an autologous replacement. While this strategy enables the formation of non-immunogenic tissue, it is limited by poor control over tissue thickness, intrinsic thrombogenicity, lack of elastin, and the need for invasive procedures and long implantation times [6].

More recently, the in situ TE approach has gained increasing attention as a potentially simpler and more cost-effective strategy for producing off-the-shelf implants. This approach involves the direct implantation of a cell-free scaffold at the native valve site, leveraging the body's intrinsic regenerative capacity to recruit endogenous cells and promote tissue formation and remodeling while providing initial mechanical functionality [3,6].

Despite significant progress across these paradigms, the clinical translation of TEHVs remains limited, indicating that technological advancement alone is insufficient. Increasing evidence suggests that successful valve regeneration critically depends on how effectively engineered constructs recapitulate the multiscale principles governing native valve function, including hierarchical structure, anisotropic mechanics, mechanoresponsive cell behavior, immune regulation, and temporally coordinated remodeling.

In this review, we critically examine recent advances in tissue-engineered heart valves through the lens of biomimetics. By integrating insights from native valve biology and mechanobiology with contemporary strategies in scaffold design, bioactive functionalization, biomechanical conditioning, and emerging technologies, we aim to clarify how biomimetic principles can guide the development of functionally mature TEHVs. Furthermore, we discuss current challenges toward clinical translation and highlight future directions for achieving durable, regenerative, and clinically viable heart valve replacements. While numerous reviews address individual aspects of TEHV development (materials, cells, or bioreactors), fewer integrate mechanobiology, immune regulation, and scaffold architecture as a coupled system. This review specifically addresses how failure to synchronize these dimensions underlies limited clinical translation.

2. Native Heart Valve Structure and Pathophysiology

2.1. The Anatomy of Healthy Heart Valves

Human heart valves are highly specialized, dynamic structures whose primary function is to ensure unidirectional blood flow through more than 100,000 opening and closing cycles per day, and approximately 3 billion cycles over a lifetime [11–13]. The human heart contains four valves—two atrioventricular valves (AV), the mitral and tricuspid valves—that separate the atria from the ventricles, and two semilunar valves (SL), the aortic and pulmonary valves, that separate the ventricles from the great arteries [4,12]. Each valve is tailored to the hemodynamic conditions of its anatomical location. The AV consist of two (mitral) or three (tricuspid) leaflets, with external supporting chordae tendineae that attach the leaflet to papillary muscles within the ventricles. In contrast, the SVs comprise three leaflets termed cusps and lack an external chordae and papillary muscles [4,11].

In diastole, the papillary muscles are relaxed, and high pressure in the atrium causes opening of the mitral (left) and tricuspid (right) valve leaflets to promote blood flow into the respective ventricle. Once ventricular pressure increases, the AV leaflets close and maintain coaptation to prevent eversion of the valve leaflets into the atria. As the ventricle contracts, blood exits through the open SL and the ventricle relaxes to begin the cycle again [11].

The architecture of the valves reflects their functional demands. The leaflets and cusps are composed of a stratified and anisotropic extracellular matrix (ECM) organized into three distinct layers [4,11,12]:

- The fibrosa (~45%), located on the outflow side, is composed of densely collagen fibers (mainly type 1), aligned circumferentially. This layer confers tensile strength and resistance to high transvalvular pressures, especially in the aortic and mitral valves, during valve closure. Due to the high degree of collagen alignment, the fibrosa represents the primary load-bearing layer of the leaflet.
- The spongiosa (~35%), forms the middle layer, which accommodates relative motions between adjacent layers. It is composed mainly of proteoglycans (primarily Versican)

and glycosaminoglycans (GAG) including hyaluronan, which provide viscoelastic damping, resist compression and contribute to leaflet flexibility.

- The ventricularis (or atrialis) (~20%), on the inflow side, is dominated by elastin fibers aligned radially, facilitating stretch and retraction, enabling rapid leaflet opening and restoration of leaflet geometry after each cardiac cycle.

This layered ECM structure ensures that stiffness and flexibility are finely balanced across different loading conditions during the open closure cycle [12]. Importantly, leaflet architecture varies between valves to match their pressure and loading environment: the left-sided valves (aortic and mitral) endure significantly higher pressures than the right-sided valves. As a result, the aortic and mitral valves are structurally more robust than their right-sided counterparts [12].

The ECM components of the valve leaflet are populated by valvular interstitial cells (VICs) and encapsulated by an overlying single layer of valve endothelial cells (VECs). VICs are distributed throughout the ECM and are primarily responsible for ECM synthesis, turnover and repair, acting as dynamic regulators of matrix composition and mechanical integrity. This is achieved by a balanced secretion of matrix degradation enzymes, including matrix metalloproteinases (MMPs), and their inhibitors (tissue inhibitor of metalloproteinase TIMPs). On the other hand, VECs form a tight and functional barrier between the blood and the inner valve tissue, protecting it against the physical and mechanical stress of the hemodynamic environment, preventing inflammation and thrombosis. In addition, VECs have been shown to communicate molecularly with VICs by sensing hemodynamic forces and translating them into biochemical signals that regulate VIC behavior [5,11,12].

2.2. Mechanobiology of Native Valves

Native heart valves are exposed to a complex system of mechanical forces, which vary spatially and temporally throughout the cardiac cycle. These include [11,12]:

- Shear stress, generated by blood flow over the leaflet surface, which is laminar and unidirectional on the ventricularis/atrialis layer but oscillatory and disturbed on the fibrosa layer [14,15].
- Tensile stress, experienced during valve closure, especially affects the fibrosa layer.
- Cyclic flexural loading, inherent to the repetitive opening and closing cycles, that challenges leaflet durability and requires elastin-mediated resilience.

Both VICs and VECs are highly mechanoresponsive. VECs respond to shear stress by regulating nitric oxide production, adhesion molecule expression, and secretion of paracrine mediators that influence VIC phenotype [5,16,17]. VICs, in turn, remodel the ECM in response to cyclic strain, balancing matrix synthesis and degradation via MMPs and TIMPs. In health valves, VICs are in a quiescent state. However, during growth, remodeling, or injury, VICs can transiently differentiate into an activated myofibroblast-like phenotype, characterized by proliferation, apoptosis and expression of α -smooth muscle actin. This activated state enables ECM deposition and tissue remodeling [5]. Disruption of this finely tuned mechanobiological balance, promotes maladaptive remodeling. Thus, mechanobiology represents not only a cornerstone of normal valve homeostasis, but also a key driver of pathological processes when physiological mechanical forces are varied.

2.3. Pathological Remodeling

Valvular heart disease arises when the tightly regulated structure–function–mechanobiology balance of the valve is disrupted, leading to maladaptive remodeling of leaflet tissue [11,12]. Abnormal mechanical loading, genetic predisposition, and inflammatory stimuli can trigger pathological changes in extracellular matrix organization and cellular phenotype. These processes are commonly associated with three major remodeling outcomes: myxomatous degenera-

tion, characterized by proteoglycan accumulation and collagen disruption; fibrotic remodeling, involving excessive collagen deposition and leaflet stiffening; and calcification, in which VICs undergo osteogenic differentiation and deposit mineralized nodules within the ECM [11,12].

At the cellular level, pathological remodeling is largely driven by sustained VIC activation and dysregulated matrix turnover, ultimately compromising leaflet mechanics, durability, and valve function.

3. Biomimetic Strategies in Scaffold Design

Building on the structural organization, mechanobiological behavior, and failure mechanisms of native heart valves, biomimetic scaffold design aims to translate these features into well-defined and controllable engineering strategies. Heart valves are characterized by a highly complex and articulated structure, upon which valve leaflet function critically depends. The primary design objective for TEHVs is therefore to mimic the native tissue microstructure, while matching native mechanical proprieties and anisotropy [4], ensuring adequate strength, flexibility and durability to endure the cyclic stresses and strains of the cardiovascular environment. Accordingly, scaffold design and properties play a crucial role in the success of both in vitro and in situ TEHVs [6]. One of the key considerations in the design of polymeric scaffolds for TEHVs is the biodegradation profile. Ideally, degradation byproducts should be biocompatible and non-toxic, and the rate of degradation should be coordinated with new tissue formation, thereby preserving structural integrity while providing support for progressive tissue formation, for in situ TEHVs [3,6,7].

If the scaffold degrades too rapidly, valve function may be compromised, and infiltrating cells may lose the guiding template for regeneration. On the other hand, excessively slow scaffold degradation may induce prolonged inflammatory responses and impair remodeling [5,6]. In addition, scaffolds should exhibit appropriate porosity and interconnectivity to favor cell infiltration, nutrient transport and neovascularization. They should also provide nano- and microscale cues capable of regulating cell adhesion, proliferation and phenotype, while ensuring hemocompatibility and resistance to calcification, to minimize thrombosis and mineral deposition. Biomimetic design strategies may involve the selection of scaffold materials, architectural features, cell types, or fabrication techniques, each targeting specific aspects of native valve structure and function.

3.1. Scaffold Materials

In both native and engineered environments, a reciprocal relationship exists between cells and the extracellular matrix (ECM); biomaterials influence cellular behavior through their chemical and mechanical properties, while cells actively remodel the surrounding microenvironment. Consequently, tissue-engineered heart valve (TEHV) scaffolds must simultaneously withstand cyclic hemodynamic loading and facilitate long-term biological integration. Current research focuses on two primary categories: decellularized extracellular matrix (dECM) and artificial polymeric scaffolds [3,4,6].

While dECM—derived from allogenic or xenogenic donors—provides high structural and compositional fidelity, its clinical utility is often hindered by donor scarcity and potential immunogenic risks. These limitations have prompted a shift toward synthetic and natural polymers, which offer a more scalable and tunable alternative. Polymeric scaffolds facilitate precise control over shape, porosity, and mechanical behavior, and can be further functionalized into “smart” biomaterials that direct specific cell adhesion and differentiation via bioactive ligands [3,4,6].

- dECM Scaffolds maintain native architecture but require rigorous decellularization to minimize immunogenicity, which can subsequently compromise mechanical integrity [3,4,6].

- Polymeric Scaffolds provide an essentially unlimited supply and controlled degradation kinetics, though they often require surface modifications to match the intrinsic bioactivity of native tissue [3,4,6].

3.1.1. Decellularized ECM

Decellularized extracellular matrix (dECM) scaffolds offer high structural fidelity but face significant translational hurdles. While decellularization protocols were designed to reduce immunogenicity and improve host cell infiltration, xenogenic valves have encountered clinical failures due to inflammation, calcification, and poor recellularization. In contrast, allogenic valves demonstrate superior hemodynamic performance and cell survival, yet their application is constrained by donor scarcity [6,18–27].

To mitigate the mechanical deterioration often associated with decellularization, recent post-processing strategies, such as temporary polyvinyl alcohol (PVA) encapsulation, have successfully enhanced the tensile strength, handling, and suturability of porcine matrices without compromising cytocompatibility. For instance, Chen et al. [28] investigated the PVA as a dynamic encapsulation layer for decellularized porcine heart valves. They demonstrated that PVA-treated decellularized porcine heart valves exhibited significantly increased tensile strength and elastic modulus compared to untreated controls, effectively counteracting the typical mechanical deterioration caused by decellularization. The PVA layer also stabilized the valve structure during suturing procedures. After PVA removal, the modified valves retained improved mechanical integrity and supported robust adhesion and proliferation of human umbilical vein endothelial cells *in vitro*, confirming excellent cytocompatibility. These findings suggest that controlled post-processing approaches may mitigate some of the mechanical limitations associated with decellularization.

Despite these advancements, the success of dECM remains highly dependent on precise immunomodulation and remodeling control, motivating the development of more tunable synthetic alternatives [29].

3.1.2. Synthetic Polymers

Synthetic polymers are widely used in TEHV scaffold fabrication due to their tunable mechanical properties and degradation kinetics. Common materials include polyglycolic acid (PGA), polycaprolactone (PCL), polylactic acid (PLA), poly L-lactic acid (PLLA), and poly(glycerol sebacate) (PGS), all offering controlled performance under physiological loading conditions [3,4,13,28,30–33].

PGA and its copolymers are among the most established materials because of their high mechanical strength and predictable degradation profile, often combined with polymers such as P4HB or PLLA to better match tissue regeneration timelines [34]. PCL is biocompatible and easily processed, with mechanical properties adjustable through molecular weight and fabrication parameters [35]. PGS, an elastomeric polymer, degrades more rapidly (4–6 weeks) and allows modulation of mechanical behavior through curing conditions. Electrospun PGS:PCL composites have demonstrated anisotropic properties comparable to native valve tissue and supported robust cell growth in preclinical models [32].

PLA and PLLA exhibit slower hydrolysis due to more stable ester bonds, with degradation spanning 12–24 months or longer [34]. These materials are frequently combined with biological matrices to balance durability and remodeling capacity. For example, PLA-reinforced sandwich scaffolds have significantly enhanced tensile performance while maintaining endothelial compatibility [36].

Degradation kinetics vary substantially: PGA typically resorbs within months (often ~8 weeks *in vivo*), PGS within weeks, PLA/PLLA over 1–2 years, and PCL over 1–3 years

under physiological conditions. Matching degradation rates to tissue regeneration remains essential in TEHV design [3].

Overall, synthetic polymers provide precise control over scaffold architecture and mechanics but often require biofunctionalization to enhance cell adhesion and long-term remodeling.

3.1.3. Natural Polymers

Natural polymers, including fibrin, collagen, gelatin, hyaluronic acid (HA), chondroitin sulphate (CS), alginate, and polyhydroxyalkanoates such as P4HB, have been widely explored for TEHV scaffolding [3,4]. These biomaterials more closely mimic the extracellular matrix (ECM), providing a favorable microenvironment for cell adhesion, migration, and matrix deposition. However, due to limited mechanical robustness, natural polymers are most commonly used as hydrogels or cell carriers in hybrid systems combined with synthetic frameworks [3,4].

Fibrin is one of the most frequently applied natural polymers in TEHVs. Its degradation occurs through enzymatic cleavage by plasmin and matrix metalloproteinases (MMPs), and can be tuned by adjusting fibrinogen/thrombin concentrations or adding protease inhibitors [3,4]. Kreinin et al. investigated fibrin clot formation in prosthetic valves using a 3D-printed pulsatile aortic valve model and demonstrated that fibrin deposition strongly depended on valve orientation and local flow conditions, with increased clotting in low-shear recirculating regions, supported by complementary CFD analysis [37].

Collagen, the primary structural component of the fibrosa layer in native valves, is highly cytocompatible and exhibits low antigenicity, making it an ideal candidate for TEHV scaffolds [3,4]. Ma et al. developed a biomimetic trilayer construct composed of atelocollagen, HA, and a fibrillar elastin gel to reproduce leaflet anisotropy (Figure 1). Their scaffold exhibited hydration-dependent nonlinear stress–strain behavior and physiologically relevant bending mechanics, closely resembling native valve tissue [38].

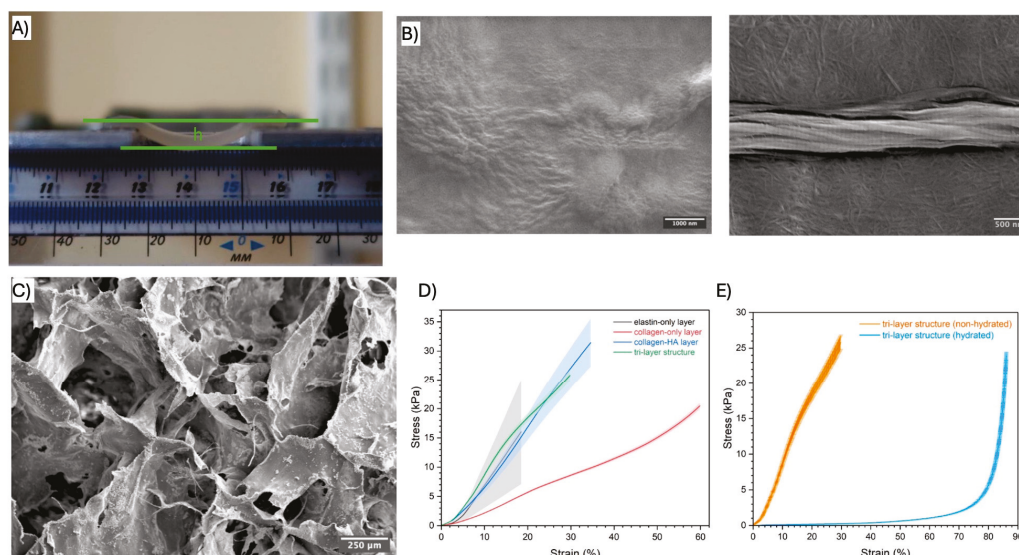


Figure 1. (A) Hydrated scaffold showing deflection measurements. (B) SEM images of the collagen suspension before (left) and after (right) fibrillogenesis. The left image lacks distinct fibers, whereas the right image reveals fibrillar atelocollagen in the background and the characteristic D-spacing pattern along the central fiber bundle formed post-incubation. (C) SEM image of crosslinked collagen-only scaffolds at 50 $\times$ magnification. (D) Compressive stress–strain curves for the trilayer scaffold and its individual component layers in the non-hydrated state; shaded regions indicate standard deviation. (E) Compressive stress–strain curves for the trilayer scaffold comparing non-hydrated and hydrated states, with error bars representing standard deviation. Adapted from Ma et al. [38], Exploration of BioMat-X, 2024, licensed under CC BY 4.0.

Gelatin, a denatured derivative of collagen, is commonly modified into gelatin methacrylate (GelMA), which can be UV-crosslinked into hydrogels with tunable properties. Xu et al. [14] combined melt-electrowritten anisotropic PCL scaffolds with GelMA/chitosan methacrylate hydrogels (Figure 2), producing composites that matched native leaflet mechanics, supported valvular interstitial cell growth, and improved hemocompatibility. In vivo implantation further showed reduced immune activation and suppressed calcification compared to PCL-only scaffolds.

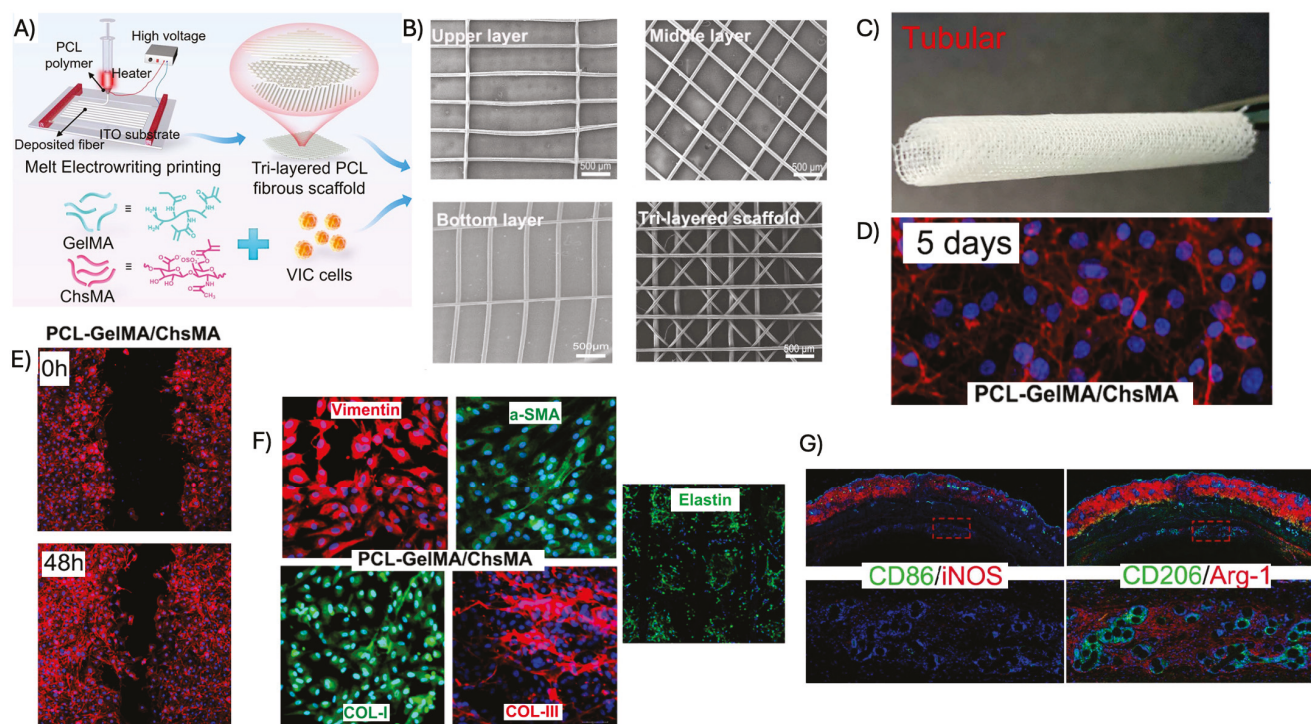


Figure 2. (A) Fabrication of a biomimetic heart valve tissue-engineered (HVTE) scaffold using MEW-enabled tri-layered PCL combined with 3D cell culture in bioactive hydrogels (GelMA/ChsMA). (B) Fiber organization in the tri-layered MEW-PCL scaffold: transversely aligned fibers on the top layer, diamond-shaped fibers in the middle, and vertically aligned fibers on the bottom; includes SEM image of the assembled scaffold. (C) Photograph of the tubular tri-layered MEW-PCL scaffold. (D) F-actin and DAPI staining of HUVECs cultured on scaffold surfaces after 5 days. (E) Scratch wound healing assay at 0 and 48 h. (F) Expression of Vimentin, α -SMA, COL-I, COL-III, and Elastin in VICs 3D-cultured within PCL-GelMA and PCL-GelMA/ChsMA scaffolds. (G) Representative immunofluorescence images after 4-week implantation, showing CD86/iNOS for M1 macrophages and CD206/Arg-1 for M2 macrophages. Red frame indicates the zoomed area displayed on the bottom. Adapted from Xu et al. [14], *Journal of Nanobiotechnology*, 2024, licensed under CC BY 4.0.

Hyaluronic acid (HA), a glycosaminoglycan naturally present in valve ECM, regulates hydration and cell behavior [3,4]. Lei et al. demonstrated that incorporating HA into fibrin scaffolds altered microstructure and reduced leaflet retraction forces by ~25%, addressing a key failure mode associated with excessive myofibroblast contraction [39].

Overall, natural polymers offer superior biocompatibility and intrinsic cell-instructive properties compared to synthetic materials. However, their limited mechanical strength and often rapid degradation generally prevent their use as standalone valve scaffolds. As a result, natural polymers are most effectively incorporated into composite or hybrid designs, where they complement synthetic components to achieve an optimal balance of biological functionality, mechanical stability, and controlled degradation, more closely emulating native heart valve tissue. Scaffold material selection therefore requires careful consideration

of both immediate structural demands and long-term regenerative potential. To support this comparison, Table 1 summarizes the key properties of the most prominent polymer systems investigated for TEHV applications.

Table 1. Comparative assessment of primary material classes for tissue-engineered heart valve (TEHV) scaffolds, detailing trade-offs between biological fidelity and engineering control. The evaluation encompasses decellularized extracellular matrix (dECM), synthetic polymers, and natural polymers based on their biocompatibility, degradation kinetics, and mechanical resilience under physiological hemodynamic loading.

Material Classes	Advantages	Disadvantages
Decellularized ECM (dECM) (e.g., Porcine/bovine valves)	- Superior structural fidelity. - Retains native ECM composition. - High initial integrity and native anisotropy.	- Risks of residual immunogenicity. - Potential for calcification - Decellularization process can compromise tensile strength.
Synthetic Polymers (e.g., PGA, PCL, PLA, PGS)	- High tensile strength. - Able to withstand physiological hemodynamic loads. - Tunable degradation kinetics (rapid to slow).	- Limited intrinsic bioactivity. - Requires surface modification for cell adhesion.
Natural Polymers (e.g., Collagen, Fibrin, GelMA, HA)	- High biological compatibility. - Provides an optimal niche for tissue regeneration. - Susceptible to natural remodeling (MMPs).	- Relatively low mechanical performance - Typically requires reinforcement with synthetic backbones.

3.1.4. Hybrid Materials

Hybrid scaffolds that combine synthetic and natural polymers have emerged as a valid strategy in TEHV design, as they synergistically exploit the mechanical robustness and tunable degradation of synthetic frameworks with the bioactivity and cell-instructive properties of natural matrices. In these constructs, synthetic polymers such as PGA, PCL, or PLLA provide structural integrity and anisotropic reinforcement, while natural components such as collagen, fibrin, gelatin, or hyaluronic acid enhance cell adhesion, migration, and ECM remodeling [3,4].

Several studies highlight the advantages of this approach. Agnieszka et al. demonstrated that PGA/P4HB scaffolds enable tailored degradation while maintaining mechanical stability suitable for valve regeneration [6]. Similarly, Masoumi et al. reported electrospun PGS:PCL trilayer scaffolds achieving biomimetic anisotropy and functional leaflet motion in an ex vivo porcine model [32]. More recently, Xu et al. combined melt-electrowritten PCL architectures with GelMA-based hydrogels, producing composites that matched native leaflet mechanics while reducing immune activation and calcification in vivo [14].

3.2. Scaffold Architectural Design

Beyond material composition, scaffold architecture plays a decisive role in translating material properties into functional valve behavior. The architectural properties of scaffolds play a key role in mechanical properties, cellular attachment, growth, alignment and consequently construct functionality [6]. To facilitate physiological valve function, TEHVs aim to replicate the native tissue architecture, which is characterized by pronounced anisotropy and a multilayered organization. Fibrous and hydrogel scaffolds have been extensively employed to mimic the structural features of native heart valves, characterized by the fibrosa, ventricularis/atrialis and spongiosa layers which are enriched in collagen, glycosaminoglycans, and elastin fibers, respectively.

Importantly, scaffold morphological properties—such as fiber alignment, fiber diameter, pore size, and overall porosity—strongly influence cell–biomaterial interactions,

mechanical performance, and degradation behavior [40]. As a result, scaffold architecture (Figure 3) represents a critical design variable in achieving biomimetic valve function.

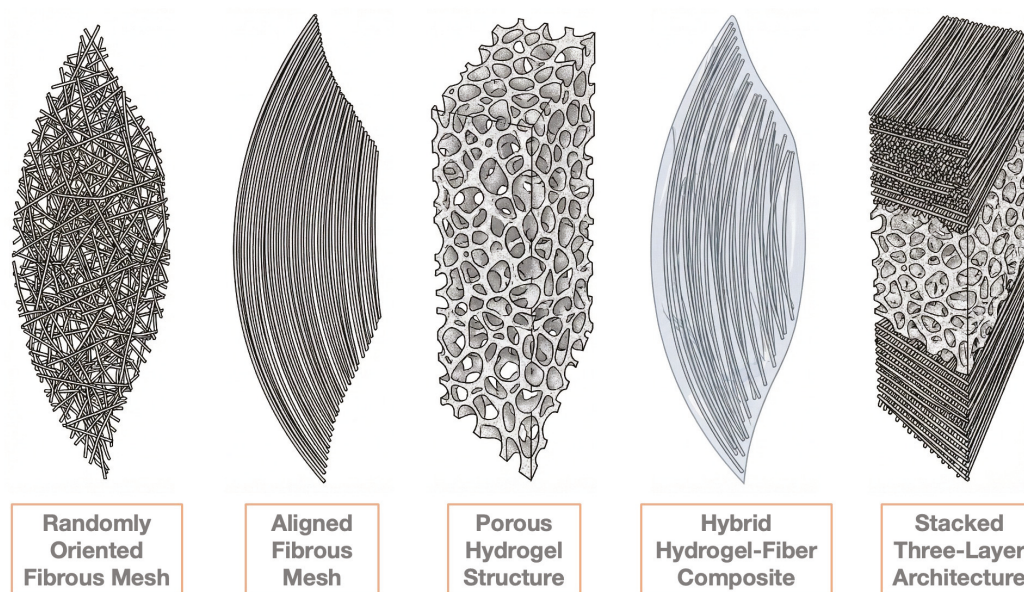


Figure 3. Schematic illustration of scaffold architectural designs impacting TEHV function. From left to right: randomly oriented fibrous meshes provide isotropic strength and suture retention but lack native anisotropy; aligned fibrous scaffolds mimic circumferential collagen organization to improve physiological anisotropic mechanics; porous hydrogel structures utilize natural polymers to create a hydrated niche for cell migration and extracellular matrix (ECM) secretion; hybrid hydrogel–fiber composites combine structural synthetic fibers for load-bearing durability with bioactive hydrogels to enhance hemocompatibility; biomimetic trilayered designs replicate native leaflet stratification (fibrosa, spongiosa, ventricularis/atrialis) to achieve nonlinear mechanical behavior and optimize cell–matrix interactions across different layers.

3.2.1. Fiber-Based Scaffolds

Fibrous scaffolds have been widely employed in TEHVs fabrication, using both randomly and directionally oriented fibers. Randomly oriented fibrous scaffolds are selected due to their relatively isotropic mechanical strength and ability to provide adequate suture retention strength in both circumferential and radial directions, which is critical for valve leaflet implantation [3]. Furthermore, the morphological characteristics of fibrous scaffolds, such as fiber diameter and pore size, can be tuned by adjusting polymer concentration and other fabrication parameters [3]. Recent studies demonstrated that randomly oriented fibrous scaffolds composed of PCL maintained stability under physiological loading conditions, while supporting cellular adhesion and growth [41]. However, despite these advantages, randomly oriented fiber architectures do not accurately reproduce the anisotropic structural and mechanical properties of native tissues. This limitation may lead to suboptimal cell alignment, altered mechanotransduction, and potentially adverse tissue remodeling over time [42].

To address these limitations, aligned fibrous scaffolds have been developed to more closely mimic the circumferential alignment of collagen fibers found in the fibrosa layer of native heart valves, thereby reproducing anisotropic mechanical behavior [42]. Aligned fibrous architectures have also been combined in layered configuration to replicate the lamellar structure of native heart valve leaflets. For example, Gürbüz et al. constructed radially aligned PCL/PGS/polysulfone nanofibrous scaffolds through 3D printing and electrospinning. The resulting scaffolds mimicked the structural and anisotropic mechanical characteristics of the ventricularis layer, enhanced human umbilical vein endothelial

cell (HUVEC) viability, and induced cell alignment demonstrating the potential of these composite scaffolds for TEHV applications [43].

3.2.2. Hydrogel Structures

Hydrogel structures are often utilized in TEHVs as biocompatible layers, and occasionally as primary scaffolding materials. A critical characteristic of hydrogels is their network pore size, which significantly influences cell behavior, including proliferation, differentiation, morphology, and ECM secretion. This pore size can be tuned through variations in polymer concentration, molecular weight, or the degree of crosslinking [3]. Recent studies have demonstrated that hydrogels composed of biopolymers like gelatin and alginate can facilitate cell migration and enhance tissue regeneration when applied in TEHV constructs [44]. Moreover, the mechanical properties of hydrogel-based TEHVs can be modulated via biomechanical conditioning to achieve the mechanical stability essential for proper valve function and to mimic some mechanical aspects of native heart valve tissues [45]. In the absence of dynamic conditioning, however scaffolds made solely from hydrogels generally exhibit stiffness and ultimate tensile strength values that are significantly lower than those of native valve tissue, limiting their standalone applicability in TEHVs [44,45].

3.2.3. Hybrid Hydrogel–Fiber Structures

To combine the favorable biological properties of hydrogels with the load-bearing capacity and anisotropy of fibrous scaffolds, hybrid hydrogel–fiber architectures have been increasingly adopted in TEHV design. This strategic combination facilitates the mimicry of both mechanical and biological properties of native heart valve tissue to a considerable extent, addressing limitations associated with either material class alone. Hybrid constructs facilitate the tuning of mechanical properties by modifying various parameters, such as fiber orientation, layer number, pore size, and hydrogel composition [3]. Robinson et al. reported that electrospun polyurethane meshes combined with hydrogel coatings yielded composite valve materials with adjustable mechanical behavior, reduced platelet and bacterial adhesion, decreased calcification, and hemodynamic performance comparable to clinical bioprosthetic valves, meeting ISO 5840-2:2021 standards [45]. These findings highlight the potential of hybrid architectures to achieve both mechanical robustness and biological functionality in TEHVs.

3.2.4. Three-Layer Scaffolds

To better replicate the complex architecture of native heart valve tissue, three-layer scaffold designs have been developed. In these constructs, TEHVs typically consist of two external anisotropic fibrous layers designed to mimic the fibrosa and ventricularis/atrialis, and a central more isotropic layer composed of glycosaminoglycans (GAGs) or natural polymer hydrogels to represent the spongiosa [3,46]. Recent advancements in this area include the work of Zhang et al. who fabricated bioinspired trilayer poly(ϵ -caprolactone) scaffolds using patterned electrospinning collectors to generate distinct layer-specific architectures (Figure 4). Their scaffolds reproduced native-like microstructural features, anisotropic mechanical behavior, and nonlinear stress–strain responses characteristic of valve leaflets. In vitro studies demonstrated cytocompatibility, guided cell alignment, and proliferation, while hydrodynamic testing confirmed effective orifice area and regurgitation rates compliant with ISO 5840-2 standards [47]. Collectively, these results underscore the relevance of trilayer scaffold architectures in approximating native valve mechanics and function.

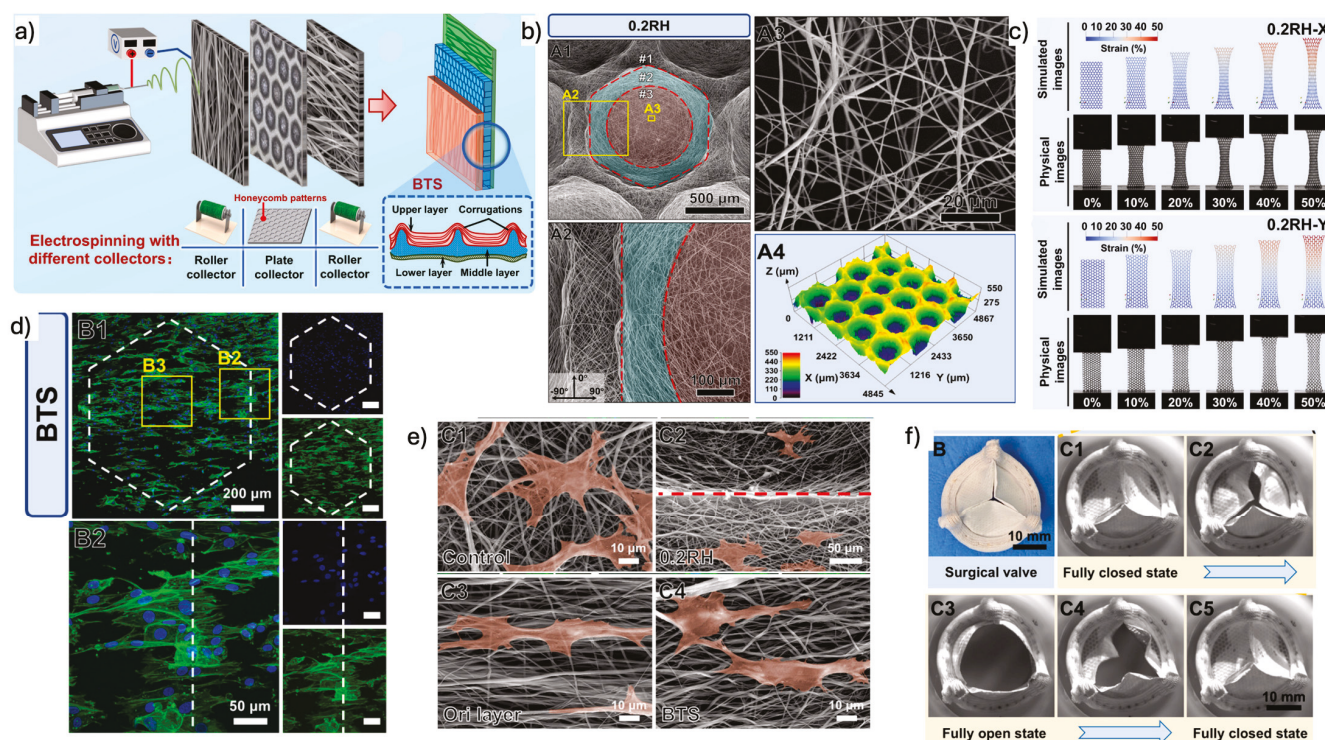


Figure 4. (a) Schematic of the fabrication of the upper, middle, and lower layers with distinct fiber morphologies via electrostatic spinning using different collectors, and the assembled biomimetic trilayer scaffold (BTS). (b) Top views of fibrous membranes and their distinct layers (#1–#3) with various honeycomb patterns (A1–A4: 0.2RH). (c) Finite element meshes for 0.2RH-X (hexagon diagonal) and 0.2RH-Y (hexagon opposite edges) showing displacement (mm) along the loading direction at strains from 0 to 50%, obtained via simulation. (d) Fluorescence images of A7r5 cells cultured on BTS fiber membranes for 3 days (green: cytoskeleton; blue: nuclei), B2 and B3 indicate border and central zooms respectively on the honeycomb pattern, only B2 is shown for simplified visualization. (e) SEM images of A7r5 cells (highlighted in red) on (C1) control, (C2) 0.2RH, (C3) Ori layer, and (C4) BTS fiber membranes. (f) Physiological hydrodynamic testing of the assembled pulmonary valve (B): valve opening and closing states throughout a heartbeat cycle (C1–C5). Adapted from Zhang et al. [47], *Chemical Engineering Journal*, 2025, licensed under CC BY 4.0.

3.3. Cell Types

The success of tissue-engineered heart valves (TEHVs) relies not only on mimicking the structural and mechanical properties of the native ECM but also on the proper selection of cell types for scaffold seeding. The selected cells must replicate the functional and structural roles of native heart valve cells while maintaining ECM synthesis, remodeling, mechanical adaptability, and signaling [4]. Among the various cellular candidates investigated for TEHV applications, valvular interstitial cells, valvular endothelial cells, and human umbilical vein endothelial cells have been most extensively studied.

Valvular interstitial cells (VICs) represent the predominant population within valve leaflets and are distributed throughout all layers of the tissue. They exhibit remarkable phenotypic heterogeneity, sharing characteristics with both fibroblasts and smooth muscle cells (SMCs) [4]. The ability of VICs to produce ECM proteins, including collagen, GAGs, and elastin, provides them with fibroblast-like traits. VICs can transition between a quiescent fibroblast-like phenotype and an activated myofibroblast phenotype, particularly in response to mechanical or biochemical stimuli [48]. This phenotypic plasticity is essential for normal valve growth, remodeling, and repair. However, sustained or excessive activation of VICs can lead to pathological remodeling, including fibrosis and calcification. Consequently, scaffold designs that accurately replicate the anisotropic and viscoelastic

mechanical environment of native valve tissue play a crucial role in maintaining VICs in a physiological, quiescent state. Recent studies from De Morales et al. have demonstrated that VICs seeded on biomimetic scaffolds fabricated from decellularized ECM significantly enhance ECM remodeling and biocompatibility in TEHV constructs, supporting their effectiveness in mimicking native valve function [33]. These findings underscore the importance of matching scaffold mechanical and structural properties to native valve tissue to regulate VIC behavior effectively.

Valvular endothelial cells (VECs) line the blood-contacting surfaces of valve leaflets and play a pivotal role in maintaining homeostasis. They provide a non-thrombogenic barrier and actively regulate VIC behavior through paracrine signaling, thus modulating inflammation, thrombosis and remodeling processes [4]. VECs possess distinct morphologies and functions compared to vascular endothelial cells; for example, they position themselves perpendicular to blood flow, allowing them to withstand complex shear stress conditions. Additionally, VECs proliferate more rapidly and produce nitric oxide (NO), which aids in maintaining VIC quiescence [48]. Accordingly, scaffold architectures that support physiological shear stress exposure and appropriate endothelial alignment are critical for promoting functional endothelialization in TEHVs. Several studies have demonstrated that scaffolds engineered to favor VEC adhesion and alignment significantly improve hemocompatibility and long-term valve performance in vitro [48].

Human umbilical vein endothelial cells (HUVECs) are commonly leveraged in the development of TEHVs as they are easily isolated and cost-effective [4]. When seeded onto electrospun or otherwise fibrous scaffolds, HUVECs have been shown to form a continuous endothelial monolayer aligned with the flow direction, enhancing scaffold hemocompatibility and closely mimicking the anti-thrombogenic function of natural endothelium [4]. Although HUVECs do not fully recapitulate the phenotype of native VECs, they are frequently employed as a surrogate endothelial cell source for in vitro studies and early-stage validation of scaffold designs. Recent studies have reported that functionalization of bio-prosthetic heart valves with HUVEC-derived extracellular vesicles or growth factor-rich coatings can enhance endothelialization, reduce thrombosis, and suppress calcification, further supporting the utility of HUVEC-based strategies in TEHV development [49].

In addition to utilizing native valve cells and HUVECs, several other cell types, including fibroblasts, have been proposed for TEHV development to enhance biomimetic outcomes. Fibroblasts are crucial components of connective tissue; they are spindle-shaped cells that contribute to ECM formation and maintain tissue integrity. They are the primary cells responsible for the development and repair of connective tissue, producing a highly ordered, collagen-rich matrix that facilitates force transmission and intricate tissue deformation [4]. Dermal fibroblasts have emerged as an alternative to VICs, offering advantages such as abundant ECM production and responsiveness to mechanical and biochemical stimuli. Dermal fibroblasts carry out multiple functions, including autocrine and paracrine signaling, proliferation, migration in response to chemotactic and mitogenic cues, and the synthesis and deposition of extracellular matrix. In a study by Syedain et al., dermal fibroblasts were seeded into decellularized heart valves using a bioreactor system, and the resulting tissue-engineered heart valves, tested in a pulse duplicator, exhibited structural properties like native valves. The use of dermal fibroblasts to generate a scaffold suitable for host cell recellularization provides a promising alternative to current tissue valve replacements [50].

Recent investigations have also provided mechanistic insights into fibroblast and VIC regulation. Batan et al. demonstrated that phosphatase and tensin homolog (PTEN) expression plays a critical role in regulating VIC activation in response to matrix stiffness. Their findings showed that PTEN overexpression preserved a quiescent fibroblast-like

phenotype, whereas PTEN inhibition promoted myofibroblast activation, suggesting that pharmacological modulation of cell–matrix signaling pathways may represent a future strategy for controlling pathological remodeling in valve disease [51]. These findings highlight the importance of selecting appropriate cell types for TEHVs and underscore the need for integrating different cellular sources to achieve desired mechanical and biological properties for optimal heart valve function.

The selection of an appropriate cell source is a pivotal decision in TEHV design, balancing biological authenticity with practical constraints such as scalability and phenotypic stability. To facilitate a clearer comparison of current strategies, Table 2 summarizes the advantages and limitations of the most frequently utilized cell types, ranging from native valvular populations to cost-effective surrogates.

Table 2. Comparative evaluation of primary and alternative cell sources utilized in heart valve tissue engineering. The table outlines the physiological roles and strategic advantages of each cell type—ranging from native valvular populations (VICs and VECs) to clinically accessible alternatives (HUVECs and dermal fibroblasts)—while highlighting the limitations regarding phenotypic stability, sourcing, and pathological activation risks in the context of long-term valve remodeling.

Cell Type	Role in TEHV	Advantages	Disadvantages and Limitations
Valvular Interstitial Cells (VICs)	Primary matrix-producing cells; distributed throughout all layers.	High biological fidelity; superior production of collagen, GAGs, and elastin; essential for natural growth and repair.	High risk of pathological activation; can transition to myofibroblasts leading to fibrosis and calcification.
Valvular Endothelial Cells (VECs)	Non-thrombogenic lining; regulates homeostasis via paracrine signaling.	Maintains hemostasis; produces nitric oxide (NO) to keep VICs quiescent; specialized to withstand complex shear stress.	Highly specific phenotype; difficult to source and maintain compared to vascular endothelial cells.
Human Umbilical Vein Endothelial Cells (HUVECs)	Surrogate endothelial lining.	Easily isolated and cost-effective; forms continuous monolayers that improve hemocompatibility and prevent early thrombosis.	Does not fully recapitulate the specialized phenotype or morphology of native VECs.
Dermal Fibroblasts	Alternative matrix producer for scaffold recellularization.	Abundant ECM production; highly responsive to mechanical stimuli; offers a scalable alternative to native valve-derived cells.	Lacks valve-specific signaling; long-term behavior in the cardiac environment may differ from native VIC populations.

Beyond the selection of individual cell sources, co-culture strategies are increasingly recognized as essential for recapitulating the native cellular heterogeneity and paracrine signaling of heart valves. By spatially organizing multiple cell types—such as seeding VECs on the surface of a VIC-laden matrix—researchers can better simulate the physiological ‘crosstalk’ required for long-term homeostasis. For instance, Jesus De Morales et al. demonstrated that maturing iPSC-derived mesenchymal stem cells into VIC-like cells within a structured environment significantly promotes healthy ECM markers. Furthermore, modern hybrid approaches, such as the sandwich structures described by Zhang et al. provide the necessary architectural cues to support these heterogeneous populations, ultimately reducing pathological activation and improving hemodynamic performance [33,47].

3.4. Fabrication Techniques

Various scaffolding techniques have been utilized to fabricate TEHVs, including molding, electrospinning, jet-spinning, melt electrowriting, textile methods, and photolithography. Often, these methods are combined, such as using electrospinning in conjunction with molding to fabricate hybrid scaffolds that can better replicate the complex structure and mechanical properties of native heart valves [3].

3.4.1. Molding

Molding has emerged as a widely used technique for creating TEHV scaffolds due to its high throughput and capacity to produce complex valve-relevant geometries [3]. This approach typically involves the use of molds, with tubular or more complex shapes—such as bi- or trilayered structures—into which various materials, primarily hydrogels, can be injected [3]. Hydrogels molded into tubular structures can be transformed into bi- or trilayered valves, either by suturing concentric layers together or using a supporting frame [52]. When hydrogels are directly molded into bi- or trilayered structures, the resulting geometry depends on the mold design, typically generated via computer-aided manufacturing methods [52]. Recent studies have demonstrated that GelMA- and Poly(ethylene glycol) diacrylate (PEGDA)-based hydrogels can maintain structural integrity and allow for adequate cell infiltration when molded into valve-like geometries [33]. However, it is essential to note that scaffolds fabricated solely from cell-laden hydrogels without biomechanical conditioning often exhibit inadequate mechanical strength and limited anisotropy, limiting their ability to replicate the functionality of native heart valve tissues [53]. To address this limitation, molding is frequently combined with other fabrication methods, such as electrospinning, to enhance mechanical performance [53].

3.4.2. Electrospinning, Jet-Spinning and MEW

Electrospinning is one of the most employed techniques for producing fibrous scaffold using various natural and synthetic polymers, such as collagen, P4HB, PCL-PLLA, PCL, PGS, polyurethane (PU), and PLLA [3]. This technique generates precursor sheets or tubes that can later be transformed into bi- or trilayered structures using suturing or origami methods, or it may directly create the desired structures thanks to specialized collectors [52]. Electrospinning generates micro- to nanoscale fibers through the application of a high-voltage electric field, which draws a charged polymer jet from a spinneret toward a grounded collector. Electrospun fibrous scaffolds closely resemble the native fibrous microarchitecture of native valve ECM, making it particularly attractive for biomimetic scaffold design [53]. The orientation and deposition of fibers can be controlled by adjusting the shape and movement of the collector, allowing for the development of scaffolds with anisotropic structures and mechanical properties that mimic those of native valve tissues [52]. Adjustments in parameters such as voltage, flow rate, and polymer concentration can facilitate control over fiber diameter, morphology, and alignment. These factors significantly impact the mechanical behavior and biological responses (e.g., cellularization) of the final TEHV [33]. Moreover, electrospun scaffolds feature a high surface-to-volume ratio, enhancing cellularization both *in vitro* and *in vivo* [4]. However, cellularization in electrospun constructs tends to occur primarily on the surface, with limited infiltration due to the dense fibrous structures. Additionally, the electrospinning method has limitations concerning the control of the architecture, diameter, and orientation of fibers making scaffolds susceptible to unwanted variations caused by fluctuations in the electric field and polymer viscosity [53].

Jet spinning represents an alternative fiber fabrication technique that relies on high-speed rotational forces rather than electric fields. This method is generally more robust to

environmental fluctuations and allows improved control over fiber deposition compared to electrospinning [3]. However, jet spinning is typically limited to simpler fiber architectures and offers less flexibility in generating complex three-dimensional geometries required for TEHVs.

Melt electrowriting (MEW) combines 3D printing and melt electrospinning to offer superior control over fiber morphology and has gained increasing attention in generating fibrous scaffolds, mainly using PCL, for TEHV applications [3]. Like electrospinning and jet spinning, MEW allows for the deposition of fibers in various geometries, including cylindrical, planar, or more anatomically relevant structures [3]. Recent studies illustrate that MEW has been effective in creating scaffolds with a crimped fiber architecture, replicating the nonlinear stress–strain behavior typical of native valve tissue [3]. Despite these advantages, MEW suffers from relatively low throughput and sensitivity to processing conditions, posing challenges for large-scale manufacturing. Additionally, issues related to charge accumulation and limited microporosity might constrain cell infiltration [53–55].

3.4.3. Textile Technologies

Textile technologies, such as weaving or knitting, utilize polymeric fibers produced by methods such as electrospinning to fabricate yarns, which are then transformed into textiles. These techniques have been employed for the development of tissue sheets or tubular structures, primarily composed of synthetic polymers such as PCL, poly(ethylene terephthalate), and poly(acrylonitrile) [3]. The scaffolds generated through this method are characterized by tunable mechanical and degradation properties, mechanical anisotropy, and structural stability. Consequently, textiles have also been employed as supporting backbones to reinforce hydrogel structures combining mechanical stability with biological functionality. For example, woven PCL scaffolds have been shown to significantly enhance mechanical integrity and cell attachment when integrated into TEHV constructs [3]. Chen et al. further demonstrated that reinforcing polymeric heart valve matrices with radially oriented NiTi wires, using textile technologies, improved leaflet kinematics, reduced stress concentrations, and resulted in favorable hemodynamic performance, highlighting the potential of textile-based hybrid designs for durable valve replacements (Figure 5) [56].

3.4.4. Cell-Free Approach

While both cellularized and cell-free approaches aim for functional restoration, they offer distinct trade-offs in clinical and industrial contexts.

Cell-free (in situ) approaches are currently the preferred strategy for large-scale production and emergency clinical use due to their superior manufacturability and “off-the-shelf” availability. By eliminating the need for complex, patient-specific cell harvest and expansion, acellular scaffolds—particularly synthetic polymeric or decellularized xenogenic matrices—reduce the risk of phenotypic drift and the high costs associated with prolonged in vitro culture [3,6,10,39].

In contrast, cellularized approaches (in vitro TE) remain the gold standard for scenarios requiring immediate biological integration and high-rate ECM turnover, such as in pediatric patients where somatic growth is a primary requirement. However, cellularized constructs face significant safety challenges, including the risk of overactive remodeling and myofibroblast-mediated calcification. From a production standpoint, the scalability of cellularized TEHVs is limited by batch-to-batch variability and the lack of standardized maturation protocols. Consequently, while cellularized valves offer higher initial biological potential, cell-free scaffolds functionalized with bioactive cues represent a more translatable middle ground for widespread clinical adoption [3,6,10,39].

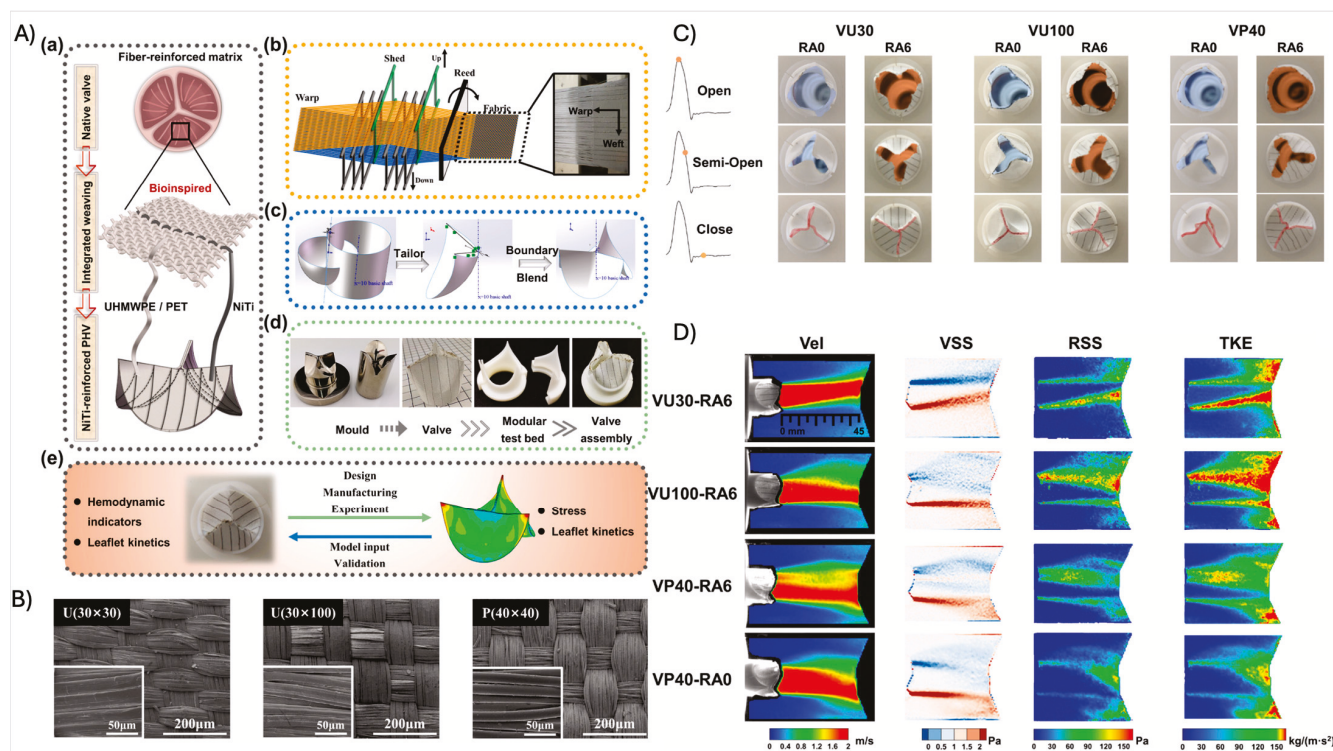


Figure 5. (A) Biomimetic design and fabrication of NiTi-TeXVal prototype. (a) Biomimetic design strategy; (b) Fabrication of integrated hybrid micro-tube; (c) Computer modeling of trileaflet polymeric valves; (d) Shaping and assembly of valve prototypes; (e) Integration of in vitro experimental research and numerical simulation to advance the field of heart valve prosthesis. (B) Characterization of leaflet materials. (C) Hemodynamic indicators and morphology of different valve designs. Images depict representative pictures of different valve designs at critical phases, including fully open during peak systole, semi-open during end systole, and fully closed during early diastole. (D) Flow field characteristics of different valve designs: contours of velocity, VSS, RSS, and TKE at peak systole downstream of the valves (about 45 mm). Adapted from Chen et al. [56], *Composites Part B*, 2023, licensed under CC BY 4.0.

3.4.5. Comparison of Fabrication Technologies

Advanced techniques, such as melt electrowriting (MEW) and nanotechnology-based approaches, offer unmatched design flexibility, but their translation to clinical-scale manufacturing requires careful consideration of feasibility and cost. MEW provides excellent reproducibility for creating anisotropic fibrous architectures [3], yet the process remains relatively slow, which may limit high-volume production. Nanotechnology strategies, including targeted drug delivery systems, enable precise functional control [56] but bring added regulatory complexity and potential challenges in maintaining batch-to-batch consistency at scale. In contrast, acellular scaffolds produced via high-speed jet spinning [39,57] are cost-effective, easier to sterilize, and suitable for “off-the-shelf” use, although they lack the immediate biological complexity of bioprinted, cell-laden constructs.

Many fabrication techniques also allow tuning of scaffold porosity, a key parameter for TEHV performance. Molding generates complex 3D hydrogel structures, with porosity largely determined by the hydrogel formulation; typical pore sizes range from 10 to 100 µm, sufficient for cell infiltration but limited by mechanical constraints [3]. Electrospinning produces fibrous scaffolds with diameters from tens of nanometers up to several micrometers, resulting in small pores (<10 µm) that can hinder cell infiltration unless modified with porogens or patterned collectors [3,52,53]. Jet-spinning, which relies on high-speed rotation rather than electric fields, improves deposition consistency and robustness but

typically produces simpler 3D architectures [3,52,53]. MEW, combining 3D printing with melt electrospinning, allows precise fiber placement and generates well-defined pores (100–300 μm) that support controlled cell behavior and tissue development. Unlike random electrospun networks, MEW scaffolds offer tunable architecture and anisotropy, closely mimicking native heart valve extracellular matrix while maintaining reproducible pore structures [3]. Finally, textile techniques, such as weaving or knitting, produce polymeric yarns with adjustable inter-fiber spacing, achieving pores of 100–500 μm that enhance mass transport and cellularization [56]. Together, these parameters can be optimized to support cell infiltration, ECM deposition, and the mechanical performance of the final construct.

Ultimately, the clinical viability of TEHVs will likely depend on hybrid manufacturing strategies that combine the high-resolution precision of MEW with the speed and cost-efficiency of traditional textile or electrospinning methods. Effective scaffold design emerges from the coordinated integration of material composition, architectural organization, cellular interactions, and fabrication techniques, rather than from any single design element alone (Figure 6).

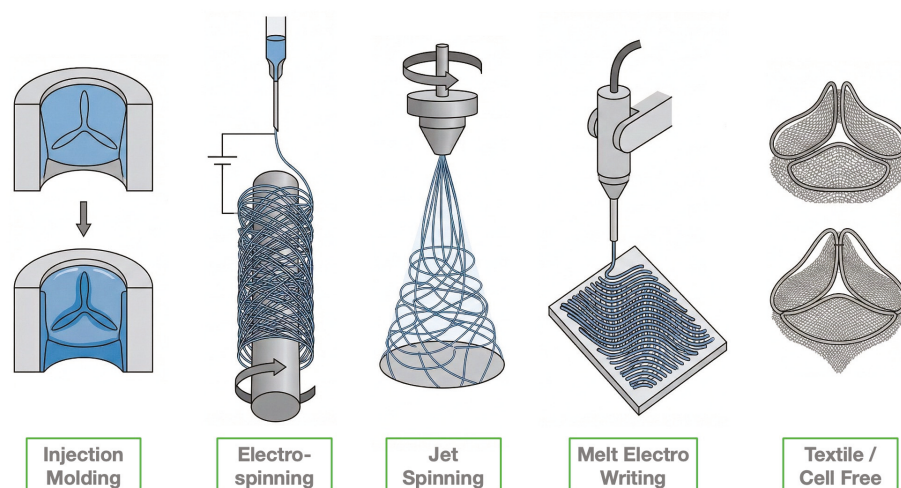


Figure 6. Overview of primary biofabrication techniques for TEHV scaffolds. From left to right: molding facilitates high-throughput generation of anatomical hydrogel geometries; electrospinning utilizes high-voltage fields to create fibrous meshes mimicking ECM microarchitecture; jet spinning (Rotary Jet Spinning) uses centrifugal force for scalable fiber fabrication; melt electrowriting (MEW) enables precise, computer-controlled deposition of microfibers for defined anisotropic patterns; hybrid/textile assembly integrates reinforced backbones (e.g., woven nitinol or polymeric fabrics) with hydrogels or electrospun layers for enhanced mechanical durability.

Summarizing, biomimetic scaffold design for TEHVs requires a careful balance of material properties, architectural features, and cellular components to replicate the structure and function of native heart valves. Decellularized ECM offers unmatched structural fidelity but is limited by donor availability and potential immunogenicity, whereas synthetic polymers provide tunable mechanical properties, degradation kinetics, and architecture, allowing precise control over scaffold porosity and anisotropy. Natural polymers contribute intrinsic bioactivity, promoting cell adhesion, migration, and ECM deposition, and are most effective when incorporated into hybrid constructs with synthetic frameworks to achieve both biological functionality and mechanical robustness. Scaffold architecture—from fibrous and hydrogel structures to hybrid hydrogel–fiber and trilayered designs—plays a pivotal role in guiding cellular organization, mechanical behavior, and tissue remodeling. The selection and spatial organization of cell types, including VICs, VECs, HUVECs, and fibroblasts, further modulate ECM synthesis, endothelialization, and long-term valve performance, with co-culture strategies enabling physiologically relevant paracrine signal-

ing. Finally, fabrication techniques such as molding, electrospinning, jet-spinning, melt electrowriting, and textile methods provide complementary approaches to tailor scaffold geometry, pore size, and anisotropy, supporting both cellularized and cell-free strategies. Taken together, these integrated design considerations highlight the importance of a multi-scale, multidisciplinary approach in developing TEHVs capable of recapitulating native valve mechanics, biological cues, and long-term functionality.

4. Biological Cues

Beyond structural and mechanical considerations, effective TEHV scaffolds must actively regulate biological interactions with the host environment, motivating the integration of bioactive cues discussed below. The ability of THEVs to grow and remodel upon implantation is strongly influenced by the host immune response. By incorporating bioactive factors into the implanted valve, it is possible to promote the recruitment of specific cell populations and modulate local cellular function [57]. In this context, biomimetic design plays a central role by reproducing the biochemical and structural cues naturally found in native heart valves. Scaffolds can effectively manage host–tissue interactions, steering them towards regeneration rather than fibrosis. Recent studies have further demonstrated that microenvironmental conditions, including biochemical gradients and biomaterial stiffness, strongly influence cell recruitment, differentiation, and functional maturation [53]. For instance, Cheng et al. showed that the maturation trajectory of pluripotent stem cell-derived cardiomyocytes is highly dependent on microenvironmental cues, highlighting the broader implications of biophysical and biochemical signaling in cardiac tissue engineering [58]. Accordingly, biological cues are increasingly integrated into TEHV designs to recapitulate key aspects of native valve biology and support constructive tissue remodeling. Antithrombotic coatings, immunomodulatory molecules, cell-interactive ligands, and drug delivery systems, that can be employed to emulate physiological processes and recreate a regenerative microenvironment, will be discussed in the subsequent sections.

4.1. Antithrombosis and Anti-Calcification Strategies

Despite advancements in biomaterial development to reduce hemolytic, toxic, and immunological reactions, thrombotic and thromboembolic complications related to cardiovascular implants remain a major clinical concern. Upon contact with blood, a thin monolayer of plasma proteins quickly covers the surface of the implanted scaffold. The composition and conformation of these proteins depend on the surface chemistry of the biomaterial including charge, roughness, and porosity. These surface properties influence platelet adhesion and activation thereby affecting thrombus formation [6,59]. In physiological valves, the endothelial layer maintains hemostasis by presenting a surface that resists nonspecific protein absorption and prevents platelet activation [6]. Consequently, a major objective of biomimetic TEHV design is to reproduce these antithrombotic properties during the early post-implantation period.

Heparin functionalization is among the most widely adopted strategies to mimic the anticoagulant activity of endothelial cells. Heparin-coated scaffolds inhibit platelet aggregation and thrombin activity while also helping to mitigate calcification, modulating macrophage activity and reducing local inflammation [60–62]. Recent research has demonstrated the effectiveness of heparin-functionalized scaffolds in reducing thrombus formation in vivo; for example, Hu et al. reported that biological valve leaflets modified with heparin-loaded hydrogels exhibited enhanced hemocompatibility, improved endothelialization, reduced immune response, and strong anti-calcification performance, compared to conventional glutaraldehyde-treated tissues [63].

4.2. Immunomodulatory Cues

Following implantation, porous scaffolds experience early infiltration by circulating immune cells, which release cytokines and growth factors that regulate cell recruitment, inflammation and tissue remodeling. Among these immune cells, macrophages play an essential role in resolving inflammation due to their ability to transition from a pro-inflammatory state (M1-like) to a reparative phenotype (M2-like) [6]. Rather than suppressing immune activation, modern biomimetic strategies aim to guide macrophage polarization toward pro-regenerative phenotypes, mirroring physiological wound healing processes.

Cytokines such as monocyte chemoattractant protein-1 (MCP-1) and stromal cell-derived factor 1 α (SDF-1 α) are illustrative examples of biomimetic signaling molecules that convert immune activation into regenerative processes. MCP-1 is known to promote the recruitment of circulating inflammatory cells, facilitating rapid and homogeneous infiltration of matrix by blood-derived cells [6,64]. Recent studies indicate that the release of MCP-1 from scaffold materials can enhance cell migration and retention, thereby boosting the regenerative capacity of implanted valves [6,65,66]. Similarly, SDF-1 α has proven crucial in recruiting blood-derived tissue-producing progenitor cells and controlling valve cell phenotype. Its incorporation into TEHV architectures has been associated with improved endothelialization and ECM remodeling [6].

In addition to soluble factors, scaffold chemistry itself can exert immunomodulatory effects. Modifications using oxidized hyaluronic acid, peptide-functionalized surfaces, or ROS-responsive hydrogels have been shown to induce macrophage polarization toward M2 phenotypes, improve hemocompatibility, and reduce calcification in vivo [6]. These findings highlight the potential of embedding immunomodulatory function directly into scaffold design rather than relying solely on exogenous factor delivery.

4.3. Cell Recruitment, Adhesion and Differentiation

In native heart valves, cellular behavior is regulated by highly specific cell–matrix interactions mediated by integrins, adhesion ligands, and growth factor gradients. Biomimetic TEHV design thus focuses on recreating these interactions by functionalizing scaffold surfaces with bioactive molecules that regulate cell recruitment, adhesion, and differentiation [6]. Antibody-based strategies, such as functionalization with anti-CD34 or anti-CD133 antibodies, have been employed to capture circulating progenitor cells and accelerate endothelialization. While these approaches can enhance early cell attachment, they often lack specificity and may recruit heterogeneous cell populations [67–69]. In contrast, peptide-based ligands derived from extracellular matrix proteins offer more selective control over cell adhesion. The RGD peptide promotes integrin-mediated attachment of multiple cell types, whereas the REDV peptide preferentially binds endothelial progenitor cells, supporting targeted endothelialization [70,71]. Liu et al. demonstrated that decellularized porcine pericardium functionalized with ulvan, REDV, and Vascular Endothelial(VE)-cadherin antibodies significantly enhanced endothelialization while reducing thrombogenicity, inflammation, and calcification, both in vitro and in vivo, highlighting the regenerative potential of multifunctional surface modifications [70].

Growth factor incorporation represents a complementary strategy for regulating cell fate. Vascular endothelial growth factor (VEGF), growth differentiation factor 11 (GDF11), and nitric oxide (NO)-releasing systems have been shown to promote endothelialization, suppress platelet activation, and modulate inflammatory signaling [72,73]. GDF11, a Transforming Growth factor (TGF)- β superfamily member, promotes endothelial progenitor cell (EPC) proliferation, migration, survival, and angiogenic activity via TGF- β /Smad signaling, and has recently been implicated in EPC recruitment in ischemic models. Its integration into biomaterials represents a novel strategy for TEHVs. In this context, scaffold

folds incorporating copper-modified GDF11 enabled controlled NO release, enhancing EPC recruitment, hemocompatibility, and endothelialization while supporting a pro-vascular microenvironment [72,73].

More broadly, growth factor incorporation within biomaterials is an effective approach to modulate the local cellular niche and mimic physiological signaling. VEGF enhances endothelial cell migration, proliferation, and progenitor recruitment, while TGF- β 1 regulates ECM synthesis and interstitial cell differentiation. Controlled immobilization, often via heparin-based strategies, ensures sustained bioactivity and spatiotemporally regulated release, thereby supporting functional tissue regeneration [6,54,74].

However, it is important to underline that reproducing these cues in a biomimetic manner requires precise spatial and temporal control to prevent excessive exposure and unintended effects.

4.4. Drug Loaded Scaffolds

A further evolution in biomimetic TEHV design involves the incorporation of drug delivery systems that locally reproduce the paracrine signaling environment of native tissue. Drug-loaded scaffolds can deliver anti-inflammatory, anti-thrombotic, or regenerative molecules in a controlled way, thereby mimicking the endogenous release of bioactive mediators following tissue injury [57,75]. However, physiological healing is a temporally dynamic process involving sequential signaling events. Consequently, biomimetic drug delivery systems must be engineered to provide spatiotemporal control over release kinetics, preventing the “burst release” phenomenon that often leads to rapid clearance and off-target effects. To achieve this, bioactive agents can be physically entrapped within scaffold fibers, chemically conjugated to polymer backbones, or encapsulated within nano- and micro-carriers dispersed within the scaffold matrix [4,6].

Recent approaches in TEHV design extend beyond growth factor delivery to include pharmacological agents that modulate cell phenotype and immune responses. For example, Simvastatin-loaded scaffolds have been shown to enhance cardiomyoblast proliferation and adhesion, while C-type natriuretic peptide (CNP) delivery suppresses pathological activation of valvular interstitial cells, preserving leaflet geometry and preventing stenosis [76,77].

Immunomodulation has emerged as a critical strategy, with controlled release of cytokines such as MCP-1 and SDF-1 α guiding macrophage polarization from pro-inflammatory M1 to reparative M2 phenotypes. This approach reduces fibrotic encapsulation and promotes constructive tissue remodeling and endothelialization [78,79].

Biocompatible polymers and injectable hydrogels further support controlled drug release and tissue integration. Materials such as poly(ϵ -caprolactone) enhance growth factor retention, while chitosan–fucoidan hydrogels delivering FGF-2 improve neovascularization and scaffold integration [34,80]. In parallel, incorporation of anti-thrombotic agents, including nitric oxide-releasing systems, mitigates platelet activation and inflammatory responses, improving scaffold hemocompatibility and durability. Multifunctional hybrid scaffolds combining antithrombotic, immunomodulatory, and anti-calcific properties have demonstrated superior endothelialization and reduced calcification in vivo [73,81,82].

Nanoparticle-based delivery systems further expand TEHV functionality by enabling targeted and sustained release of therapeutic agents. Recent studies show that nanoparticles can modulate drug pharmacokinetics and reduce off-target toxicity while supporting endothelialization and suppressing calcification, for example through the co-delivery of RunX2-siRNA and VEGF [83–85].

Overall, the integration of controlled drug delivery systems within TEHV scaffolds enables localized, biomimetic modulation of the cellular microenvironment, enhancing regen-

eration while limiting thrombosis, inflammation, and calcification, and represents a promising direction for next-generation heart valve engineering. Among the many biological cues explored (Figure 7), immunomodulation—particularly macrophage polarization—has emerged as a dominant determinant of constructive remodeling in vivo.

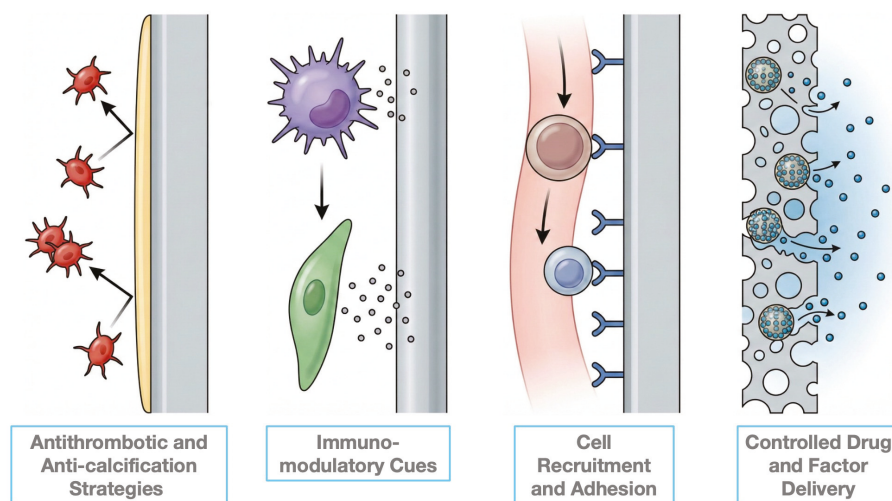


Figure 7. Strategies for biological functionalization of TEHV scaffolds to regulate host–tissue interactions. From left to right: antithrombotic surface modification (e.g., heparin coating) inhibits platelet activation and adhesion to mimic endothelial thromboresistance; Immunomodulatory signaling utilizes bioactive cues to guide macrophage polarization from pro-inflammatory (purple, spiky) to reparative (green, elongated) phenotypes; selective cell recruitment employs surface ligands (e.g., RGD or REDV peptides) or antibodies to capture circulating progenitor cells; controlled drug delivery systems, such as embedded nanoparticles, provide spatiotemporal release of growth factors (e.g., VEGF, GDF11) to promote endothelialization and remodel the microenvironment.

5. Biomimetic Bioreactors and Conditioning Systems

While scaffold design and biological cues define the regenerative potential of TEHVs, functional maturation depends on exposure to physiological environments. Heart valve disease continues to drive the clinical use of mechanical and bioprosthetic valve replacements; however, both options face limitations such as thrombogenicity, poor longevity, and a complete lack of growth potential, which is particularly problematic for pediatric patients. TEHVs have therefore emerged as a promising alternative, aiming to provide living, adaptable replacements capable of remodeling over time.

In this context, a bioreactor can be defined as a controlled *in vitro* culture device that maintains engineered tissues under regulated conditions, ensuring adequate nutrient delivery, waste removal, and environmental control. Beyond basic culture support, bioreactors are often coupled with conditioning systems, meaning the application of defined mechanical and biochemical stimuli—such as pulsatile flow, cyclic strain, and shear stress—to mimic the native valve environment and promote tissue maturation.

Central to TEHV development are therefore bioreactors and conditioning platforms designed to replicate physiological environments and deliver the cues necessary for functional tissue development. More importantly, these systems act as mechanobiological training tools, accelerating extracellular matrix (ECM) deposition, anisotropic fiber organization, and endothelial maturation prior to implantation [85,86].

Modern bioreactors are not simple culture chambers, but aim to reproduce key aspects of native valve biomechanics to guide tissue development toward functional competence. By tuning parameters such as strain amplitude, flexural frequency, and pulsatile shear stress,

conditioning protocols can be optimized to improve leaflet strength, reduce maladaptive remodeling, and enhance long-term durability [87,88].

5.1. Mechanical Conditioning Platforms

Several classes of bioreactors have been developed to mimic specific mechanical stimuli experienced by native heart valves (Figure 8), including dynamic flexure, cyclic stretch, pulsatile flow, and rotating bioreactor systems. Each platform targets distinct components of the valvular mechanical environment and contributes to tissue maturation.

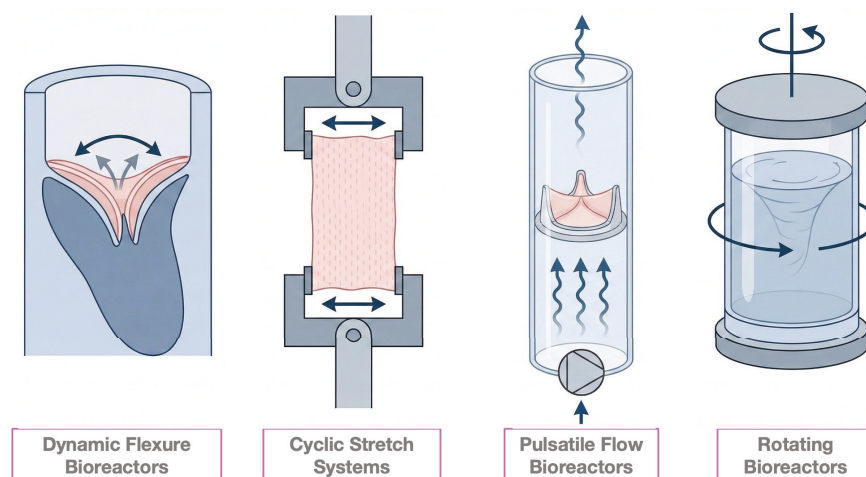


Figure 8. Schematic representation of mechanical conditioning platforms used for TEHV maturation in vitro with arrows representing the direction of applied stimuli. From left to right: Dynamic flexure bioreactors simulate repetitive leaflet bending during the cardiac cycle to promote collagen organization and bending stiffness; Cyclic stretch systems apply controlled tensile loading to stimulate extracellular matrix synthesis and enhance tensile strength; Pulsatile flow bioreactors recreate physiological hemodynamic conditions, including shear stress, to promote functional endothelialization; Rotating bioreactors utilize dynamic culture primarily to enhance mass transport and nutrient distribution throughout thick tissue constructs.

- **Dynamic Flexure Bioreactors** simulate the repetitive bending experienced by heart valves during the cardiac cycle. By subjecting scaffolds to controlled flexural deformation, they help promote the alignment of cells and matrix deposition, which are critical for the function of natural heart valves [86]. This flexural loading is particularly relevant for establishing leaflet-like collagen architecture and improving bending stiffness [86].
- **Cyclic Stretch Bioreactors** apply intermittent stretching to engineered tissues, replicating the dynamic mechanical loading that native heart valves undergo. The application of cyclic stretch has been shown to enhance cell proliferation and matrix production, facilitating the creation of stronger and more resilient tissues. Therefore, stretching parameters can be tuned to optimize tissue growth and functionality [87]. In TEHVs, cyclic strain is also a key driver of VIC differentiation and tensile reinforcement of the developing leaflet [88].
- **Pulsatile Flow Bioreactors** simulate the hemodynamic conditions of the cardiovascular system. They create a pulsatile flow, like blood flow, which encourages endothelialization and the formation of a vascular network within the graft. By controlling the flow rate, pulsatile frequency, and pressure, these bioreactors can support realistic flow conditions that promote the development of layered tissues characteristic of native heart valves [87]. Such shear-mediated conditioning is essential for generating a functional endothelial monolayer and reducing thrombogenic risk post-implantation [89,90].

- **Rotating Bioreactors** use centrifugal forces to enhance mass transport and nutrient distribution within the scaffold. Although less specific to valve mechanics, these systems are particularly useful to improve cell distribution and oxygenation, ensuring that the cells embedded in the scaffold receive adequate environmental support to proliferate and secrete extracellular matrix components necessary for tissue formation [86].

5.2. Advantages of Dynamic Conditioning

Beyond conventional mechanical stimulation, dynamic conditioning strategies are increasingly being refined to better reproduce the coupled biomechanical environment of native valve leaflets. In vivo, heart valves are exposed simultaneously to cyclic flexure, tensile stretch, and complex pulsatile shear stress, and TEHV maturation depends on integrating these stimuli rather than applying them in isolation [85,87]. Consequently, next-generation bioreactors aim to deliver multi-axial loading regimes that more accurately guide tissue remodeling toward physiologic structure–function relationships.

Dynamic conditioning has been shown to enhance collagen deposition, promote anisotropic ECM organization, and support endothelial stability, all of which are essential for achieving leaflet durability and hemocompatibility. Importantly, conditioning protocols can be tuned to avoid maladaptive responses such as excessive myofibroblast activation, tissue compaction, or premature stiffening, which remain key failure modes in TEHV development [88,90]. For instance, Dumont et al. designed a novel pulsatile bioreactor that allowed for real-time monitoring of tissue responses under conditions that precisely replicated hemodynamic forces experienced within the cardiovascular system [91]. Their findings confirmed that pulsatile flow significantly enhances endothelialization of valvular tissues, effectively reducing the risk of thrombosis—a critical challenge in valve replacement therapies. The endothelial cell coverage observed under dynamic conditions was superior compared to static cultures, reinforcing the importance of mechanical stimulation in achieving a functional endothelial layer capable of promoting patency and functionality post-implantation.

5.3. Emerging Condition Strategies

Beyond conventional mechanical stimulation, recent studies have explored the integration of additional physical cues to further enhance tissue maturation. Gabetti et al. explored the effects of electrical stimulation in conjunction with pulsatile flow on the maturation of engineered cardiac tissues, revealing synergistic effects that collectively enhanced cellular alignment, proliferation, and ECM deposition [92]. Although primarily explored in myocardial models, such approaches highlight the potential of multimodal conditioning strategies for future TEHV applications.

Advances in bioreactor design have also enabled real-time monitoring and adaptive conditioning. Ongoing research by Voß et al. emphasizes the need for sensory systems within bioreactors that can detect and adapt to the real-time conditions of the implants, tailoring the mechanical conditioning to enhance patient-specific TEHV maturation processes [93]. This adaptability can significantly improve outcomes, particularly for pediatric patients who require growth potential in engineered valves due to their continuous development [94].

It has been observed that Magnetic Resonance Imaging (MRI) and ultrasound-compatible bioreactors allow non-invasive longitudinal assessment of valve motion, ECM formation, and mechanical performance under dynamic loading [95]. A metal-free bioreactor newly described by Mohapatra et al., compatible with high-field MRI (7T) and ultrasound imaging, enabled non-invasive, longitudinal monitoring of TEHV maturation under physiological dynamic loading (pressure, flow, cyclic motion). In experiments using

textile-based biohybrid valves, conditioning in this bioreactor resulted in abundant ECM production, including collagen and α -smooth muscle actin, and formation of a coherent endothelial monolayer across the valve cusps [95]. This integration of real-time imaging with dynamic mechanical conditioning marks a critical step toward reproducible, controlled preclinical maturation protocols and potentially earlier indicators of in vivo performance. Such feedback-controlled systems may reduce batch-to-batch variability and accelerate the standardization required for clinical translation [85,90].

5.4. Integration with Computational Modeling

Computational modeling is increasingly integrated with experimental bioreactor platforms to improve the design, control, and predictive understanding of TEHV maturation. While bioreactors provide the physical stimuli required for tissue development, in silico models offer a complementary framework to quantify local mechanical environments, optimize culture parameters, and anticipate biological outcomes that are difficult to measure experimentally. In particular, computational tools can be used to predict scaffold deformation, flow-induced shear stress distributions, cell viability, and long-term tissue remodeling under different conditioning regimens, thereby reducing reliance on empirical trial-and-error approaches.

Recent finite-element frameworks, such as the model proposed by Sesa et al., enable the prediction of collagen deposition, anisotropic ECM remodeling, and valve deformation under physiologically relevant mechanical loading [96]. Such approaches provide powerful guidance for optimizing scaffold geometry, tailoring conditioning protocols, and improving pre-implantation validation. Moreover, computational studies can incorporate key design variables—including scaffold architecture, degradation kinetics, fiber orientation, and dynamic conditioning—to estimate long-term mechanical performance and maturation trajectories. Recent reviews highlight that properly engineered polyester-based scaffolds (e.g., PCL) may remain structurally stable for up to two years in vivo, providing a sufficient temporal window for endogenous tissue formation and integration [97].

Overall, the combination of biomimetic bioreactors and computational modeling strengthens TEHV development by enabling a more rational and reproducible maturation strategy. By capturing the interplay between hemodynamic forces, cyclic deformation, and scaffold–cell interactions, modeling enhances control over ECM deposition, fiber alignment, endothelialization, and differentiation. Static culture alone is no longer defensible for TEHVs intended for clinical translation, and the integration of predictive simulations with adaptive conditioning systems represents a critical step toward durable, functional, and scalable living valve replacements.

6. Emerging Technologies

New technologies are increasingly expanding TEHV development by building on advances in biomimetic scaffolds, biological cues, and mechanical conditioning. Among the most promising emerging approaches are 3D bioprinting, smart biomaterials, and image-guided tissue engineering, combined with digital twin frameworks, all aiming to improve the precision, and translational potential of engineered valves.

3D bioprinting enables layer-by-layer assembly of spatially controlled cell-laden constructs allowing patient-specific geometries that approximate native leaflet architecture. Customized alginate-based hydrogel valve scaffolds, for example, have demonstrated reproducible leaflet-like geometries suitable for in vitro evaluation [98] (Figure 9). However, current biopinks often lack sufficient mechanical strength and require post-printing conditioning or hybrid reinforcement to achieve functional competence.

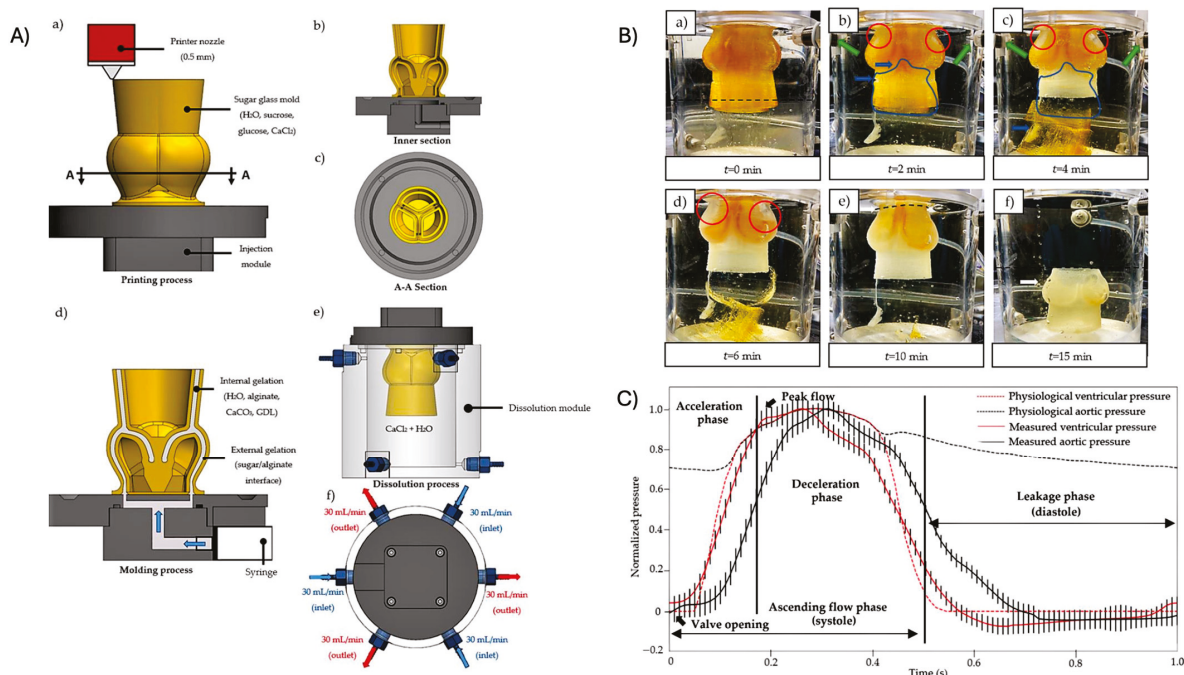


Figure 9. (A) Schematic of aortic valve scaffold fabrication. (a) Sugar glass aortic valve mold loaded with CaCl₂ and printed using a 0.5 mm nozzle and 0.2 mm layer height. (b) Inner section of the mold. (c) A–A cross-section showing the interior at the mid-sinus. (d) Molding of the scaffold by syringe injection along the direction indicated by the arrow of a sodium alginate formulation containing GDL and CaCO₃ for internal gelation; external gelation occurred at the sugar–alginate interface. (e) Transfer of the injected mold to the dissolution module. (f) Sugar mold dissolution using a 90 mL/min total (inlet/outlet) flow of water supplemented with CaCl₂. (B) Dissolution of the sugar glass mold and final scaffold gelation. (a) $t = 0$ min, fresh CaCl₂ solution above the dashed line and dissolved sugar at the bottom (yellowish liquid). (b) $t = 2$ min, inlet jets of fresh CaCl₂ (green arrows) created holes in the sugar glass mold (red circles); blue arrows and outlined regions indicate dissolving inner structures falling to the bottom. (c) Mold dissolution at $t = 4$ min. (d) Mold dissolution at $t = 6$ min. (e) $t = 10$ min, scaffold manually cut with a scalpel at the dotted line. (f) $t = 15$ min, dissolution and gelation completed. (C) Average measured pressures (aortic and ventricular) normalized ($n = 12$ cardiac cycles) at 60 BPM. These were obtained using a custom-made cardiac bioreactor on three aortic valve scaffolds and compared to the physiological profiles. The vertical bars represent the standard deviation at each acquisition time (100 Hz) of the average profiles of the three aortic valve scaffolds. Adapted from Rioux et al. [98], *Int. Jour of Mol-Scienc.*, 2022, licensed under CC BY 4.0.

In parallel, smart biomaterials introduce stimulus-responsive functionality into scaffold systems. These engineered polymers or composites respond to physiological cues (e.g., pH, enzymatic activity, oxidative stress), enabling adaptive regulation of tissue remodeling. Targeted nanoparticle systems have shown potential in reducing calcification in preclinical models [99]; however, long-term safety, manufacturing reproducibility, and regulatory complexity remain key barriers to clinical translation.

Finally, image-guided tissue engineering integrates advanced imaging modalities with computational modeling to assess valve dynamics and guide scaffold optimization. When coupled with digital twin frameworks, these approaches enable predictive simulations of patient-specific hemodynamics and durability [100]. Although still emerging, such strategies may facilitate predictive, patient-tailored TEHV design.

Following the analysis of these emerging strategies, Table 3 provides a comprehensive synthesis of recent TEHV research milestones and biofabrication advances, highlighting the ongoing paradigm shift toward hybrid, multilayered constructs capable of combining immediate mechanical performance with long-term biological remodeling.

Table 3. A comprehensive summary of recent advancements in tissue-engineered heart valve (TEHV) research, detailing the specific type of valve reconstructed, the materials utilized, the fabrication processes employed, the biological components (cells and bioactive compounds) investigated, and the primary in vitro or in vivo highlights reported for each study.

Authors (Year)	Type of Valve Reconstructed	Type of TEHV	Materials Used	Scaffold Architecture	Fabrication Process	Cells Used	Bioactive Compounds Added	Drugs Loaded	Tissue Maturation Strategy	Study Type	Highlights
Bai et al. (2025) [82]	Hybrid TEHV	Hybrid TEHV	Decellularized xenogenic heart valve, oxidized HA, SF-PBA, curcumin	Glycoprotein-like network	One-pot hybridization	N/A	Curcumin	Yes (Curcumin acts as both)	N/A	In vivo	Enhanced antithrombosis, immunomodulation, and reduced calcification post-implantation.
Chen et al. (2023) [28]	Pulmonary Valve	Atrial/Pulmonary Valve	Polyvinyl alcohol, porcine tissue	Dynamic template	Decellularization, PVA encapsulation	HUVECs	N/A	N/A	Post-treatment cytocompatibility achieved	In vitro	Improved processability and mechanical properties of decellularized valves treated with PVA.
Chen et al. (2023) [56]	Heart valve (NiTi-TexVal)	Synthetic Polymeric Heart Valve (PHV) intended as a proof-of-concept for future TEHV applications	Nitinol (NiTi) wire, Polyethylene glycol terephthalate (PET), and Ultra-high molecular weight polyethylene (UHMWPE)	Fiber-reinforced textile composite with NiTi wires oriented in the radial direction within a plain weave fabric matrix	Integrated micro-tube weaving technology followed by thermoforming/molding and 3D-printed assembly	N/A	N/A	N/A	N/A	In vitro experimental research (pulsating flow, PIV) and numerical simulation (Finite Element Analysis)	Mimicking natural collagen, slashing peak stress by 46.15% and ensuring consistent leaflet motion. PEI outperformed UHMWPE during the cardiac cycle.
Jesus De Morales et al. (2024) [33]	Trileaflet aortic valve	3D-bioprinted, cell-laden hybrid scaffold	Gelatin methacrylate (GelMA), Polyethylene glycol diacrylate (PEGDA), and Polylactone (PCL)	Multilayered: PCL base sheet (100 µm) reinforced with cell-laden GelMA/PEGDA hydrogel	Extrusion-based 3D bioprinting (layer-by-layer)	iPSC-derived mesenchymal stem cells (iMSCs) matured into Valvular Interstitial-like (VIC-like) cells	N/A	N/A	Dynamic conditioning (shear and stretch) for 14 days to promote ECM synthesis	In vitro (Dynamic culture/bioreactor)	Hybrid scaffold mimics the native aortic valve's trileaflet structure and mechanics. Dynamic culture significantly reduced ACTA2 (indicator of disease/activation). Promoted healthy ECM markers (Vimentin, Collagen I, Aggrecan) over 14 days.

Table 3. Cont.

Authors (Year)	Type of Valve Reconstructed	Type of TEHV	Materials Used	Scaffold Architecture	Fabrication Process	Cells Used	Bioactive Compounds Added	Drugs Loaded	Tissue Maturation Strategy	Study Type	Highlights
Fan et al. (2026) [101]	Decellularized aortic valve (DAV)	In situ regenerative/immunomodulatory TEHV	DAV, TPA-PVA hydrogel, mesoporous silica nanoparticles (MSNs)	Natural DAV matrix coated with nanoreservoir-laden hydrogel	Decellularization and hydrogel-nanoreservoir surface modification	THP-1 (macrophages), HUVECs (endothelial cells)	Folic acid, Hyaluronic acid	CY-09 (NLRP3 inhibitor)	In situ M2 macrophage polarization	In vitro and In vivo (rat models)	Intelligent ROS-responsive drug release; Induced M2 macrophage polarization; Accelerated endothelialization and remodeling.
Ma et al. (2024) [38]	Heart Valve Leaflet	Trilayer Biomimetic Scaffold	Atelocollagen (Type I), Hyaluronic Acid (HA), Elastin gel	Trilayer structure mimicking the native leaflet's structure, composition, and anisotropic bending	Fabrication/Characterization	N/A	N/A	N/A	N/A	Characterization/In vitro (aim)	Scaffold mimics the structure, composition, and anisotropic bending properties of native heart valve leaflets.
Motta et al. (2023) [102]	FibraValves (Nanofiber Heart Valves)	Synthetic nanofiber-based heart valve replacement	PLCL (Poly(L-lactide-co-ε-caprolactone)) and PCL (Polycaprolactone)	Micro- and nanofiber scaffolds with anisotropic alignment mimicking native valve mechanics	Focused Rotary Jet Spinning (FRJS)	Host cells (in vivo)	None	None	In situ re-modeling (host-mediated)	In vitro testing and acute in vivo deployment	FibraValves (Nanofiber Heart Valves)
Rioux et al. (2022) [98]	Tricuspid aortic valve	Sodium alginate hydrogel scaffold	Sodium alginate and sugar glass sacrificial molds (sucrose, dextrose, calcium chloride)	Native-like tricuspid geometry featuring sinus of Valsalva and sino-tubular junction	3D-printed sacrificial sugar glass molding and dissolution	None in this study (intended for future autologous cell seeding)	None in this study (intended for future ECM protein addition)	None	None (intended for dynamic cardiac bioreactor stimulation)	In vitro Biofabrication and hydrodynamic validation	This method enables the rapid manufacturing of complex tricuspid scaffolds in 25 min with high geometric reproducibility and functional performance mimicking physiological flow profiles.

Table 3. Cont.

Authors (Year)	Type of Valve Reconstructed	Type of TEHV	Materials Used	Scaffold Architecture	Fabrication Process	Cells Used	Bioactive Compounds Added	Drugs Loaded	Tissue Maturation Strategy	Study Type	Highlights
Wang et al. (2024) [36]	Heart Valve (Aortic)	Nanofiber-reinforced Sandwich Structure	Poly(lactic Acid (PLA))/Decellularized Porcine Heart Valve (DPHV)	Sandwich Composite: PLA nanofibers/DPHV/PLA nanofibers	Electrospinning (combined with non-woven processing)	HUVECs	Decellularized ECM (natural components)	N/A	In vitro cell culture and assessment of biocompatibility	In vitro	Sandwich structure improved tensile strength by 108% and tensile strain by 571%; optimized spinning time promoted HUVEC proliferation.
Xu et al. (2024) [14]	Heart Valve	Anisotropic Scaffold	PCL/GelMA/ChsMA Hydrogels	Anisotropic PCL scaffold integrated with bioactive hydrogels	Melt electrowriting (MEW)	Valvular Interstitial Cells (VICs)	ChsMA (for hemocompatibility/endothelialization)	N/A	3D culture to foster ECM remodeling of VICs	In vitro/Mechanical testing	MEW created a biomimetic anisotropic PCL scaffold with tunable mechanical properties; ChsMA improved the hemocompatibility and endothelialization.
Yu et al. (2023) [85]	Heart Valve	Biofunctionalized DTEHV	DTEHV/Mesoporous Silica Nanoparticles (MSNPs)	Decellularized ECM	Biofunctionalization/Drug Loading onto DTEHV	N/A	VEGF (Vascular Endothelial Growth Factor)	RunX2-siRNA	Controlled release of therapeutics to inhibit calcification	Implied In vitro/Pre-clinical	Used MSNPs for controlled, simultaneous release of VEGF and RunX2-siRNA to combat calcification.
Zhang et al. (2025) [47]	Surgical heart valve	Bioinspired trilayer tissue-engineered heart valve (TEHV) scaffold	Poly(ϵ -caprolactone) (PCL)	Trilayer structure with native-like corrugations and orthogonal fiber alignment	Electrospinning using roller and honeycomb-patterned collectors	A7r5 (smooth muscle cells) and HUVECs (endothelial cells)	None reported	None reported	In vitro cell culture for alignment and proliferation	In vitro experimental research and finite element modeling	This biomimetic trilayer scaffold successfully replicated the native “J-curve” stress-strain behavior and mechanical anisotropy of natural leaflets. Hemodynamic testing confirmed the valve meets ISO 5840-2 standards for effective orifice area and regurgitation.

Key to abbreviations: BHV (bioprosthetic heart valves); BP (Bisphosphonate); ChsMA (chitosan methacryloyl); Cu (Copper ions); DAVs (Decellularized Aortic Valves); DHV (decellularized heart valves); DPHV (decellularized porcine heart valves); DPP (decellularized porcine pericardium); DPVs (Decellularized Porcine Valves); DTEHV (Decellularized Tissue-Engineered Heart Valve); ECM (extracellular matrix); FEA (Finite Element Analysis); FRIS (Focused Rotary Jet Spinning); GDF11 (Growth Differentiation Factor 11); HA (hyaluronic acid); HUVECs (human umbilical vein endothelial cells); MEW (melt electrowriting); MSNPs (mesoporous silica nanoparticles); N/A (not applicable/not available); PCL (Poly(ϵ -caprolactone)); PLA (polylactic acid); PLCL (Poly(L-lactide-co- ϵ -caprolactone)); PVA (polyvinyl alcohol); REDV (Arginine–Glutamic acid–Aspartic acid–Valine peptide sequence); ROS (Reactive Oxygen Species); RVOT (Right Ventricular Outflow Tract); SF-PBA (Silk Fibroin–Phenylboronic Acid); siRNA (Small interfering RNA); TEHV (tissue-engineered heart valve); VEGF (vascular endothelial growth factor); VICs (valvular interstitial cells).

7. Challenges Toward Clinical Translation

Despite significant progress in TEHV technologies, considerable challenges remain in their transition to clinical applications. These challenges involve technical, biological, and regulatory issues that must be solved to ensure safe and durable engineered valves.

7.1. Scalability and Manufacturing Reproducibility

One of the primary challenges concerns scalability and manufacturing reproducibility. As TEHV designs become increasingly complex maintaining batch-to-batch consistency becomes more difficult. As detailed by Ganizada et al. [103], maintaining consistency across batches while scaling up production presents risks related to mechanical properties, biocompatibility, and overall tissue function.

To overcome these barriers, the field must transition toward automated, closed-loop biofabrication platforms that integrate real-time quality control sensors. Standardizing “off-the-shelf” acellular scaffolds—such as those produced by high-throughput electrospinning or melt electrowriting (mew)—can significantly reduce the variability inherent in patient-specific cell expansion while ensuring structural stability [92,94].

Implementing well-defined protocols and standardized approaches in bioreactor design is crucial for achieving the consistent quality necessary for clinical use.

7.2. Vascularization and Integration

Vascularization and integration of TEHVs within host tissues also pose significant challenges. The ability of engineered valves to integrate with surrounding vascular networks is vital for nutrient delivery and tissue viability. Actionable strategies to accelerate this process include the functionalization of scaffolds with pro-angiogenic cues, such as vascular endothelial growth factor (VEGF) or growth differentiation factor 11 (GDF11), which have been shown to enhance endothelial progenitor cell (EPC) recruitment. Furthermore, the use of perfusion-based pre-conditioning within bioreactors can stimulate the formation of primitive vascular-like networks and maintain ECM integrity before implantation [72,73]. Recent studies emphasize that effective vascular networks must be developed within TEHVs to prevent ischemic conditions post-implantation. As demonstrated by Tomiyama et al. [104], using perfusion bioreactors that mimic physiological flows can enhance vascularization while supporting the maintenance of scaffold architecture and ECM integrity.

Without sufficient vascular networks, long-term success of TEHVs is jeopardized as they may fail to receive necessary blood supply in vivo.

7.3. Long-Term Durability and Regulatory Pathways

Ensuring the long-term durability of TEHVs in vivo remains another significant concern. Issues such as calcification, immunogenic responses, and mechanical fatigue can lead to the failure of engineered constructs under physiological conditions. A comprehensive understanding of the long-term performance of TEHVs is necessary for advancing these technologies into the clinical arena. To address these failure modes, future roadmaps prioritize hybrid architectures that combine the mechanical robustness of synthetic polymers like polycaprolactone (PCL) with the biological resilience of natural hydrogel coatings, such as chitosan methacryloyl (Chs-MA), which have been shown to suppress calcification and improve hemocompatibility [13]. Studies by Stefano et al. reviewed durability and compatibility issues associated with various scaffolding materials, indicating the need for developing strategies that enhance the resilience and longevity of engineered heart valves in a dynamic biological environment [105].

In addition to technical and biological considerations, regulatory and ethical challenges play a major role in limiting clinical translation. Navigating regulatory and ethical hurdles further significantly complicates the clinical translation of TEHVs. Regulatory bodies require extensive data on safety and efficacy from both preclinical and clinical trials before approving these innovative therapies for patient use. TEHVs often combine advanced biomaterials, living cells, bioactive molecules, and dynamic conditioning systems, resulting in complex products that do not fit neatly within existing regulatory frameworks. The lack of standardized benchmarks for preclinical testing, conditioning protocols, and long-term performance metrics further complicates regulatory approval pathways, increasing development time and cost [106]. To streamline regulatory approval, the development of “in silico clinical trials” using digital twins—virtual representations of patient-specific anatomy—can provide standardized, predictive performance data for large “virtual patient cohorts,” potentially reducing the burden of costly animal testing. Establishing consensus-based benchmarks for dynamic conditioning—specifically aligned with ISO 5840 standards for effective orifice area and regurgitation—is a critical next step for the field to ensure performance metrics are met before human trials [45,95].

From a surgical perspective, scaffold suturability and anchoring remain critical translational requirements. TEHV constructs must provide sufficient suture retention strength to prevent leaflet tearing or delamination during implantation, particularly for hydrogel-rich or rapidly degrading materials. Hybrid reinforcement strategies are therefore often necessary to ensure operative handling and long-term fixation [22].

Although conductive biomaterials are gaining attention in broader cardiovascular tissue engineering, their functional relevance in TEHVs is still emerging and requires further investigation [20].

In summary, although emerging TEHV technologies show strong potential, addressing challenges related to scalability, vascularization, regulatory pathways, and long-term durability will be essential for successful clinical translation. Achieving this goal will require coordinated advances in biomaterials, manufacturing strategies, preclinical evaluation, and regulatory alignment.

8. Future Directions

Despite significant advances in tissue-engineered heart valve research—particularly in biomimetic scaffold design, dynamic conditioning systems, and improved preclinical models—the field has yet to achieve the level of functional maturity and long-term reliability necessary for widespread clinical application. Current biomimetic approaches often suffer from an incomplete recapitulation of the native valve’s complex hierarchical anisotropy, particularly the interplay between the three distinct layers (fibrosa, spongiosa, and ventricularis). Furthermore, while scaffolds can provide initial mechanical functionality, precisely controlling long-term remodeling behavior and preventing pathological outcomes—such as calcification or leaflet retraction due to excessive myofibroblast activity—remains a major hurdle. Evidence synthesized in the recent literature highlights that biomechanical stimulation, scaffold architecture, and cell–matrix dynamics are inseparable components of successful valve engineering [107]. Future progress will depend on the convergence of these elements into standardized, reproducible, and patient-tailored manufacturing strategies [108].

A key emerging trend is the transition from static scaffolds toward adaptive, mechanoresponsive architectures. Adaptive or mechanoresponsive architectures refer to scaffolds designed to dynamically adjust their mechanical properties in response to applied forces or cellular activity, thereby promoting tissue development that mimics the native valve’s biomechanical environment. Intelligent bioreactors are advanced culture systems equipped

with real-time sensors and automated control of mechanical and biochemical cues, allowing for continuous monitoring and dynamic adjustment of tissue conditioning to optimize maturation [109]. Emerging manufacturing approaches, including melt electrowriting and gradient electrospinning, enable precise control over scaffold anisotropy and layered microstructures, which can closely mimic the biomechanics of native leaflets [109]. Next generation scaffolds are expected to integrate regions of tunable stiffness, controlled degradation kinetics, and microarchitectural patterns optimized for directional collagen alignment, thereby enhancing tissue functionality [110]. Coupling these designs with in situ sensors to monitor strain, leaflet bending, and hydration states could further enhance bioreactor conditioning, thereby improving the precision of tissue maturation [109].

The maturation phase of TEHVs is increasingly supported by intelligent bioreactors equipped with closed-loop control, multiparametric sensing, and non-invasive imaging. MRI- and ultrasound-compatible bioreactors have demonstrated the feasibility of monitoring, leaflet motion, ECM deposition, and tissue stiffness without disrupting culture conditions [111,112]. Multi-modal conditioning strategies, incorporating additional physical cues like electrical stimulation, may further refine this maturation process. Future platforms are expected to integrate computational models directly into bioreactor control systems enabling dynamic adjustment of mechanical stimuli based on construct development. This approach may reduce variability between laboratories and facilitate regulatory acceptance [113,114].

At the biological level, consensus on the optimal cell source strategy remains elusive. Sources such as mesenchymal stromal cells, endothelial cells, and induced pluripotent stem cell lineages all show promise; however, challenges as phenotypic drift, overactive remodeling, and inflammatory activation persist [115]. Future research must focus on developing controlled differentiation protocols and epigenetic stabilization techniques to ensure predictable cellular behaviors post-implantation [116]. Furthermore, the integration of organoid-like microtissues, lineage-restricted progenitors, or spatially localized cell populations may enhance ECM deposition and valve durability. The exploration of fully acellular scaffolds is still ongoing, with the hope that host-driven repopulation—if biomechanically guided—will yield more stable, patient-adapted tissues [115].

Another critical direction for the future is the translation toward growth-compatible pediatric TEHVs. Achieving constructs that can accommodate somatic growth without structural failure or pathological remodeling remains a significant challenge. Advances in degradable anisotropic scaffolds, controlled immunomodulation, and mechanobiology-informed conditioning may enable the development of long-term pediatric TEHVs, thereby reducing the need for repeated surgeries. Achieving constructs that can accommodate somatic growth without structural failure—specifically by matching scaffold degradation to the rate of new tissue formation—is essential to reduce the need for repeated surgeries in younger populations [117,118].

On the translational front, computational modeling and in silico trials are expected to become increasingly important. Advanced simulation tools—such as high-fidelity fluid–structure interaction models, growth and remodeling algorithms, and machine-learning-based predictive methods can help to predict how leaflets deform, where stresses are concentrated, and how fatigue may develop in response to patient-specific hemodynamics [119,120]. These digital twins offer the potential to optimize design before the first incision is ever made [113,121].

Finally, key barriers to clinical translation remain, including variability in scaffold manufacturing, limited long-term in vivo data, and the lack of standardized benchmarks for dynamic conditioning. Addressing these issues will require shared conditioning pro-

protocols, defined implantation criteria, and comparative studies to enable reliable clinical translation [122,123].

9. Conclusions

In conclusion, the development of TEHVs has moved beyond simple material selection toward a sophisticated, integrated engineering paradigm. The main take-home message of this review is that successful clinical translation requires the synchronized control of scaffold anisotropy, immune modulation, and mechanical conditioning rather than incremental material optimization. Biomimetic design serves as the cornerstone of this approach, providing the structural and biochemical cues necessary to guide host-cell behavior toward regeneration rather than fibrosis [124,125].

However, significant limitations still restrict the leap from the bench to the bedside. Current designs frequently fail to fully replicate the adaptive and regenerative properties of native valves, often leading to long-term failure modes like calcification or structural deterioration. Furthermore, key barriers such as manufacturing variability, the lack of standardized benchmarks for dynamic conditioning, and the complexity of navigating regulatory frameworks for “living” medical devices must be addressed [124,125].

Ultimately, the field is entering a convergent phase where advanced biomaterials, adaptive bioreactors, and predictive modeling are being woven into cohesive workflows. Continued progress in standardizing these technologies and ensuring their scalability will be the defining factor in providing patients with living, growing, and durable heart valve replacements.

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Abbreviations

The following abbreviations are used in this manuscript:

The ACTA2	Actin Alpha 2 (Smooth Muscle) gene (α -smooth muscle actin marker)
Arg-1	Arginase-1
AV	Atrioventricular valves
BAV	Bicuspid Aortic Valve
BHV	Bioprosthetic Heart Valve
BPM	Beats Per Minute
BP	Bisphosphonate
CC BY	Creative Commons Attribution license
CD86	Cluster of Differentiation 86
CD206	Cluster of Differentiation 206
ChsMA	Chitosan Methacryloyl (or Chitosan Methacrylate)

CNP	C-type Natriuretic Peptide
COL-I	Collagen Type I
COL-III	Collagen Type III
CS	Chondroitin Sulphate
Cu	Copper ions
CY-09	CY-09 (NLRP3 inhibitor)
DAV	Decellularized Aortic Valve
DAVs	Decellularized Aortic Valves
DAPI	4',6-diamidino-2-phenylindole
DPP	Decellularized Porcine Pericardium
DPHV	Decellularized Porcine Heart Valve
DPVs	Decellularized Porcine Valves
DTEHV	Decellularized Tissue-Engineered Heart Valve
ECM	Extracellular Matrix
EPC	Endothelial Progenitor Cell(s)
FEA	Finite Element Analysis
FGF-2	Fibroblast Growth Factor 2
FRJS	Focused Rotary Jet Spinning
GAG	Glycosaminoglycans
GDF11	Growth Differentiation Factor 11
GelMA	Gelatin Methacrylate
GenAI	Generative Artificial Intelligence
HA	Hyaluronic Acid
HUVECs	Human Umbilical Vein Endothelial Cells
iMSCs	induced Pluripotent Stem Cell-derived Mesenchymal Stem Cells
iNOS	inducible Nitric Oxide Synthase
iPSC	induced Pluripotent Stem Cell(s)
ISO	International Organization for Standardization
M1	Pro-inflammatory macrophage phenotype (M1-like)
M2	Reparative/pro-regenerative macrophage phenotype (M2-like)
MCP-1	Monocyte Chemoattractant Protein-1
MEW	Melt Electrowriting
MMPs	Matrix Metalloproteinases
MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal Stem Cells
MSNs	Mesoporous Silica Nanoparticles
MSNPs	Mesoporous Silica Nanoparticles
MVP	Mitral Valve Prolapse
N/A	Not Applicable/Not Available
NiTi	Nitinol (Nickel–Titanium alloy)
NLRP3	NLR family pyrin domain containing 3
NO	Nitric Oxide
P4HB	Poly(4-hydroxybutyrate)
PCL	Poly(ϵ -caprolactone)
PEGDA	Poly(ethylene glycol) diacrylate
PET	Polyethylene terephthalate
PGA	Polyglycolic Acid
PGS	Poly(glycerol sebacate)
PHV	Polymeric Heart Valve
PIV	Particle Image Velocimetry
PLA	Polylactic Acid
PLCL	Poly(L-lactide-co- ϵ -caprolactone)
PLLA	Poly-L-lactic Acid
PTEN	Phosphatase and Tensin Homolog

PU	Polyurethane
PVA	Polyvinyl Alcohol
REDV	Arginine–Glutamic acid–Aspartic acid–Valine peptide sequence
RGD	Arginine–Glycine–Aspartic acid peptide motif
ROS	Reactive Oxygen Species
RSS	Reynolds Shear Stress
RVOT	Right Ventricular Outflow Tract
SDF-1 α	Stromal cell-Derived Factor 1 alpha
SEM	Scanning Electron Microscopy
SF-PBA	Silk Fibroin–Phenylboronic Acid
siRNA	Small interfering RNA
SLs	Semilunar valves
SVs	Semilunar valves
SVD	Structural Valve Deterioration
TE	Tissue Engineering
TEHV	Tissue-Engineered Heart Valve
TEHVs	Tissue-Engineered Heart Valves
TGF- β	Transforming Growth Factor beta
TIMP	Tissue Inhibitor of Metalloproteinase
TIMPs	Tissue Inhibitors of Metalloproteinases
TKE	Turbulent Kinetic Energy
TPA-PVA	TPA–PVA hydrogel
UHMWPE	Ultra-high molecular weight polyethylene
VE-cadherin	Vascular Endothelial cadherin
VECs	Valve Endothelial Cells
VEGF	Vascular Endothelial Growth Factor
VHD	Valvular Heart Disease
VICs	Valvular Interstitial Cells
VSS	Wall Shear Stress

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