Biomimetics and bioinspired surfaces: from nature to theory and applications

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Insect attachment on waxy plant surfaces: the effect of pad contamination by different waxes

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Abstract
This study focuses on experimental testing of the contamination hypothesis and examines how the contamination of insect adhesive pads with three-dimensional epicuticular waxes of different plant species contributes to the reduction of insect attachment. We measured traction forces of tethered Chrysolina fastuosa male beetles having hairy adhesive pads on nine wax-bearing plant surfaces differing in both shape and dimensions of the wax structures and examined insect adhesive organs after they have contacted waxy substrates. For comparison, we performed the experiments with the same beetle individuals on a clean glass sample just before (gl1) and immediately after (gl2) the test on a plant surface. The tested insects showed a strong reduction of the maximum traction force on all waxy plant surfaces compared to the reference experiment on glass (gl1). After beetles have walked on waxy plant substrates, their adhesive pads were contaminated with wax material, however, to different extents depending on the plant species. The insects demonstrated significantly lower values of both the maximum traction force and the first peak of the traction force and needed significantly longer time to reach the maximum force value in the gl2 test than in the gl1 test. These effects were especially pronounced in cases of the plant surfaces covered with wax projections having higher aspect ratios. The data obtained clearly indicated the impact of waxy plant surfaces on the insect ability to subsequently attach to the clean smooth surface. This effect is caused by the contamination of adhesive pads and experimentally supports the contamination hypothesis.

Introduction
It has been shown in numerous experimental studies that insects possessing hairy adhesive pads (i.e., specialized tarsal attachment devices) are able to establish a highly reliable contact and adhere successfully to a great variety of substrates having both smooth and microrough topographies [1-3]. However, in cases of waxy plant surfaces, where the plant cuticle is covered by...
micro/nanosopic three-dimensional (3D) epicuticular wax projections, insects usually fail to attach to [4-6]. The reducing effect of such plant surfaces on insect adhesion has been shown for many plant and insect species using various experimental approaches, from direct behavioral observations and simple inversion [7] or incline [8] tests up to precise measurements of attachment forces with different experimental techniques, such as pulling [9] and centrifugal [10] setups. It has been demonstrated that not only the presence of wax projections on the plant cuticle surface, but also their size, distribution, and density (number per unit area) influence insect attachment [11,12].

As an explanation for reduced insect adhesion on waxy plant surfaces, several contributing mechanisms have been previously suggested, such as (1) specific micro/nanosorousness created by wax projections (roughness hypothesis), (2) contamination of insect adhesive pads by plant wax during the contact (contamination hypothesis), (3) absorption of the insect pad secretion by the wax coverage (fluid absorption hypothesis), (4) hydroplaning induced by dissolution of the wax in the pad fluid (wax dissolution hypothesis), and (5) detached wax particles forming a separation layer between insect pads and the plant surface and serving as a kind of lubricant (separation layer hypothesis) [7,13].

To date, several experimental studies have been performed to test the first three hypotheses. As for the roughness hypothesis, it was revealed in centrifugal and pulling tests with some insect species bearing hairy attachment pads and mostly artificial substrates having different surface roughness. Insects showed several times higher attachment forces on both smooth and rather coarse microrough surfaces (>3 μm asperity size) compared to force values on 0.3 and 1 μm rough surfaces, where the range of asperity dimensions corresponded to that of typical plant wax projections [1,14-19]. This great reduction in the adhesion force was explained by the strong decrease of the real contact area between the micro/nanosorous surface profile and the tips of tenent setae covering insect adhesive pads, which are responsible for establishing an intimate contact with the surface [14].

The fluid absorption hypothesis assumes that because of the high capillarity of the 3D wax coverage, the adhesive fluid may be absorbed from the insect pad surface. The ability to absorb oil, which is one, in beetles possibly even the main, component of the pad secretion [20-22], has been demonstrated experimentally for the wax coverage in the carnivorous plant Nepenthes alata Blanco (Nepenthaceae) [23]. Force measurements of the beetle Coccinella septempunctata (L.) (Coleoptera, Coccinellidae) on microporous substrates able to absorb both polar (water) and non-polar (oil) fluids clearly showed a strong reduction of the attachment force on these substrates compared to reference smooth solid substrates [24]. The latter result has been explained by absorption of the fluid from insect adhesive pads by porous media and/or the effect of surface roughness. Because of the more elaborate experimental design (three additional force measurements on the solid sample after the test on the porous substrate), a later study with the beetle Harmonia axyridis (Pallas) (Coleoptera, Coccinellidae) proved the primary effect of absorption of the insect pad secretion by the porous substrate on the insect attachment force [25].

According to the contamination hypothesis, wax projections can completely or partially detach from the plant surface and adhere to the insect pads covered with the fluid secretion. Such contamination may diminish the attachment ability of the pad. Several previous studies performed with some coleopteran and dipteran species (both having hairy adhesive pads) have reported on grooming behavior of test insects after walking on waxy surfaces of Eucalyptus nitens (H. Deane & Maiden) Maiden (Myrtaceae) [26] and N. alata [27]. Both earlier and rather recent studies gave direct indications that 3D waxes of the plant species from the genera Brassica (Brassicaceae) [8,28,29] and Nepenthes [30-33] contaminated insect adhesive pads. Also our previous investigation of twelve waxy plant surfaces verified the contaminating ability of plant waxes, which differed among test plant species depending on the micromorphology, primarily dimensions and shape, of the wax projections [34].

The effect of geometrical parameters of wax projections on their fracture behavior, which in turn determines their contamination ability, was examined using a theoretical mechanical approach [35]. It was demonstrated that during contact formation between insect pads and a plant surface, the wax projections having very high slenderness ratio (i.e., aspect ratio) may easily buckle because of buckling, whereas other projections only in some cases fracture by bending.

To date, a very few experimental studies carried out with insects and waxy plant surfaces could confirm only indirectly the contamination hypothesis. Thus, inversion tests performed with the beetle Chrysolaena fastuosa Scop. (Coleoptera, Chrysomelidae) having hairy adhesive pads on various (among them twelve waxy) plant substrates have shown that Acer negundo L. (Aceraceae) stems reduced the further attachment ability of beetles for a certain amount of time, whereas other waxy plant surfaces either did not affect or impaired insect attachment only for a very short period of time [7]. The follow-up study on the contamination of insect pads by plant waxes explained the above effect in a more quantitative way [34].
The aim of this study was to experimentally examine how the contamination of insect adhesive pads by the plant wax contributes to the reduction of insect attachment on waxy plant surfaces and to the subsequent long-term reduction of their attachment ability. We measured the traction forces of C. fastuosa male beetles on nine waxy plant surfaces and a reference smooth glass substrate. The experimental design included two force measurements on glass (before and just after experiment on the plant surface) to test whether there is an effect of the plant surface on the ability of insects to subsequently attach to the smooth surface. If there was such an effect, the contamination of pads by the plant wax had a primary effect on the force reduction. Contaminability of insect pads by waxes of different plant species was visualized in an additional experiment.

Results and Discussion

Waxy plant surfaces

The plant surfaces studied are densely covered by different types of epicuticular wax projections depending on the plant species (Figure 1). Both ribbon-shaped polygonal rodlets in A. negundo (Figure 1a) and apical filamentous branches of tubules in B. oleracea (Figure 1d), although differing greatly in size (length ca. 20 μm in A. negundo according to [7,34] and 2 μm in B. oleracea according to [19,36]), show very high aspect ratios (ca. 100 [34] and ca. 33 [19,36], respectively). These wax structures have relatively small contact area with the underlying cuticle (A. negundo) or with wax tubules (B. oleracea). Cylindrical wax tubules in both A. vulgaris (Figure 1c) and C. majus (Figure 1e) are almost the smallest (<1 μm long [7,34]) structures with the lowest aspect ratios (3–5 [34]) among...
the plant species studied. As these projections are oriented at various angles in relation to the underlying cuticle, the contact area with the latter also varies. Flat, plate-like membranous (A. vera) or irregular (C. album, L. germanica, L. serriola, and T. montanum) wax platelets (Figure 1b,f–i), exhibiting intermediate values for both dimension and aspect ratio (0.6–1.7 μm and 9–22, respectively [7,34]), are arranged more or less perpendicularly to the surface. Because of such an arrangement, these platelets could achieve rather firm contact with the underlying cuticle using their whole thin side. Additionally, there are differences in distribution of the wax features. While in L. serriola, groups of platelets form clearly distinguishable clusters called rosettes (Figure 1h), the wax projections in other plant species are dispersed rather uniformly and almost completely cover the surfaces.

Data on the wax morphology are in line with our previous studies [7,34] for all plant species except B. oleracea, whose projections have been classified as terete rodlets. In later publications [19,36], where cryo-SEM was applied for the examination of plant surfaces, these projections were considered as round or angular tubules with dendrite-like branches on their tops. In the present study, we follow the latter opinion and treat B. oleracea wax projections as tubules bearing apical filamentous branches. Data on the dimension and aspect ratio given here for this plant species are related only to the branches, which are usually exposed to the environment, but not to the whole tubules.

Attachment organs of the Chrysolina fastuos a male beetle

General morphology

The tarsus of C. fastuos possesses two distally located claws and adhesive pads situated on the ventral side of three (out of five) proximal tarsomeres (later referred to as basal, middle, and distal) (Figure 2a,b). In common with most beetles from the family Chrysomelidae [37], this species has hairy tarsal adhesive pads (according to [1,38]). Tenent setae of these pads have different shapes of the tip: (1) a flat discoidal terminal element in mushroom-like setae situated in the central part of the basal and distal tarsomeres (only in males, present in all legs); (2) a flat and widened end plate called spatula in setae located around the field of the mushroom-like setae and in the distal part of the middle pad; and (3) a pointed sharp tip in all setae of the middle pad and in the periphery of the basal and distal pads (Figure 2b).

Recent detailed experimental studies on different beetle species, such as Leptinotarsa decemlineata Say, Gastrophyssa viridula De Geer, Chrysolina americana L. (all Chrysomelidae), C. septempunctata, and H. axyridis (both Coccinellidae) showing a distinct sexual dimorphism in structure and attachment performance of adhesive pads [15,17,24,25,39–42], as well as on mushroom-shaped contact elements of artificial attachment systems [43,44], revealed a strong adaptation of the discoidal tips to long-term adhesion on smooth substrates, especially needed for firm attachment of males to smooth female elytra during mating. Setae with spatula-shaped or pointed tips are better adapted to short-term temporary adhesion and locomotion on various microrough surfaces.

Contamination of insect pads by plant wax material

As well as in our previous study [34], we considered here only the discoidal setal tips allowing for (1) easier visualization of the contamination and (2) more precise evaluation of the degree of contamination. After insects have walked on various waxy plant substrates, adhesive pads demonstrated contamination of the setal tips by wax material in all cases (Figure 3 and
Figure 3: SEM micrographs of the ventral view of the first (basal) proximal tarsomere in Chrysolina fastuosa male beetles after they have walked on various plant waxy substrates: Acer negundo (a), Aloe vera (b), Aquilegia vulgaris (c), Brassica oleracea (d), Chelidonium majus (e), Chenopodium album (f), Iris germanica (g), Lactuca serriola (h), and Trifolium montanum (i). Scale bars: 20 μm.

Figure 4). Depending on the plant species, contamination differed in the texture of adhered wax (more or less homogeneous or structured to different extents) and in degree of contamination. Both parameters describing the contamination degree, such as the portion of setal tip surface covered with contaminating wax and the portion of setae contaminated by wax, differed significantly among the plant species used and positively correlated with each other [34]. The degree of pad contamination was higher in the tests with plants having larger dimensions and higher aspect ratios of the wax projections; however, the correlation between these two factors was non-significant in both cases ($P = 0.068$ for dimension and $P = 0.059$ for aspect ratio) [34].

Beetle attachment
Figure 5 shows typical force–time curves obtained from one beetle individual in a set of tests on reference glass gl1 (Figure 5a), waxy plant surface (Figure 5b), and in the second experiment on glass gl2 (Figure 5c). Using such curves, the maximal traction force $F_{\text{max}}$, the value of the first peak of the traction force $F_{\text{peak1}}$, and the time $T_{\text{Fmax}}$ needed to reach the maximum traction force value were measured (Figure 5a).

Values of $F_{\text{max}}, F_{\text{peak1}},$ and $T_{\text{Fmax}}$ were compared among different surfaces inside the experimental set (gl1 vs plant for $F_{\text{max}}$ and gl1 vs gl2 for $F_{\text{max}}, F_{\text{peak1}}, T_{\text{Fmax}}$) for data on all test
Figure 4: SEM micrographs of the ventral view of discoidal tips in exemplary mushroom-shaped setae of the first (basal) proximal tarsomere of Chrysolina fastuosa male beetles in clean (a) and contaminated conditions after the beetles have walked on various plant waxy surfaces: Acer negundo (b), Aloe vera (c), Aquilegia vulgaris (d), Brassica oleracea (e), Chelidonium majus (f), Chenopodium album (g), Iris germanica (h), Lactuca serriola (i), and Trifolium montanum (j). Note differences in the degree of contamination and in the texture of adhered wax depending on the plant species. Scale bars: 2 μm.

Considering force data obtained in experiments with different plant species, we found that in all plants studied, the waxy surface significantly reduced the maximum traction force $F_{\text{max}}$ compared to that produced in the first experiment on glass gl1 (Table 1). The force reduction varied greatly between plant species ranging from ca. 12-fold in C. album to over 30-fold in C. majus (Figure 7a). The comparison of the maximum traction force values $F_{\text{max}}$ between the first gl1 and second gl2 experiments on glass showed significant differences only in the experiments with A. negundo, B. oleracea, and T. montanum (Figure 7b and Table 1), where force values were lower in the second experiment on glass g2. The first peak of the traction force $F_{\text{peak1}}$ was significantly lower in the second gl2 experiment than in the first gl1 experiment on glass in the cases of A. negundo, B. oleracea, and L. serriola (Figure 7c and Table 1), whereas the difference was not significant in experiments with other plant surfaces. Regarding the time needed to reach the maximum traction force $T_{F_{\text{max}}}$ in the first gl1 and second gl2 experiments on glass, only in the case of I. germanica, it was significantly shorter during the second experiment on glass gl2 (Figure 7d and Table 1); for all other plants, this time was not significantly longer.

Thus, the comparison of the maximum traction forces $F_{\text{max}}$ obtained here from C. fastuosa males on nine waxy plant surfaces with those measured in the first experiment on the reference glass gl1 demonstrated the anti-adhesive properties of the wax coverage in the studied plant species. This effect was clearly seen when we compared data (maximum traction force values...
Figure 5: Exemplary force–time curves obtained from one beetle individual in a set of tests on the following surfaces: reference glass gl1 (a), plant (b), and glass gl2 (performed immediately after the test on plant) (c). Here, results for beetle no. 3 tested on an Acer negundo waxy stem are presented. $F_{\text{max}}$, maximal traction force; $F_{\text{peak1}}$, value of the first peak of the traction force; $T_{\text{Fmax}}$, time needed to reach the $F_{\text{max}}$ value.

Figure 6: Maximum traction force $F_{\text{max}}$ (a), first peak of the traction force $F_{\text{peak1}}$ (b), and time $T_{\text{Fmax}}$ needed to reach the maximum traction force (c) obtained on waxy plant surfaces and in the first and second experiments on glass. Data on all insects (i.e., from experiments with all plant surfaces) are pooled together. gl1, the first experiment on glass; gl2, the second experiment on glass; plant, waxy plant surfaces.

$F_{\text{max}}$ and the first peak of the traction force $F_{\text{peak1}}$, and significantly longer times $T_{\text{Fmax}}$ that the insects needed to reach the maximum traction force value, in the second experiment on glass gl2 compared to the reference (i.e., the first experiment on glass gl1) in all insect individuals and all waxy plant surfaces tested (pooled data). These results show the reduced ability of insects to subsequently attach to a smooth surface after having a previous contact with a waxy plant surface. In combination with our SEM data on contaminated beetle feet, the above outcomes of the force tests indicated that the contamination of pads by the plant wax is responsible for the attachment force reduction on waxy plant surfaces and has a short-term effect on the subsequent attachment to a smooth surface.

The comparison of experimental data among the plant species demonstrated certain differences between the species. Waxy surfaces of A. negundo and B. oleracea caused a decrease in both force values (maximum traction force $F_{\text{max}}$ and the first peak of the traction force $F_{\text{peak1}}$). In these plants, wax projections have highly elongated shapes and exhibit the highest aspect ratios among the plant species studied [19,34,36]. As these wax projections have rather small contact area with the underlying plant surface, they may wholly detach from it and, consequently, easily cause heavy pad contamination. Moreover, according to [35], such wax structures may also readily brake during contact formation with insect pads and contaminate them. Interestingly, it has been previously reported that the A. negundo stem surface diminished the further attachment

\[ F_{\text{max}} \] on all test insects and all plant species pooled together (ca. 24-fold reduction in average) as well as data obtained from five insects tested on each plant surface separately (from 12-fold to over 30-fold reduction). Our results are in line with previously reported findings in many plant and insect species [4-6].

The contaminating ability of plant waxes has been previously shown for many plants [8,28-34]. Our study clearly revealed the effect of pad contamination by plant wax material as an important mechanism of insect attachment reduction on waxy plant surfaces. First, contamination of insect pads by wax was verified for all plant species studied here. Second, we obtained significantly lower values of both the maximum traction force $F_{\text{max}}$ and the first peak of the traction force $F_{\text{peak1}}$, and significantly longer times $T_{\text{Fmax}}$ that the insects needed to reach the maximum traction force value, in the second experiment on glass gl2 compared to the reference (i.e., the first experiment on glass gl1) in all insect individuals and all waxy plant surfaces tested (pooled data). These results show the reduced ability of insects to subsequently attach to a smooth surface after having a previous contact with a waxy plant surface. In combination with our SEM data on contaminated beetle feet, the above outcomes of the force tests indicated that the contamination of pads by the plant wax is responsible for the attachment force reduction on waxy plant surfaces and has a short-term effect on the subsequent attachment to a smooth surface.

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Figure 7: Maximum traction force $F_{\text{max}}$ (a, b), first peak of the traction force $F_{\text{peak1}}$ (c), and time $T_{F_{\text{max}}}$ needed to reach the maximum traction force (d) on the waxy plant surface (a) and in the second experiment on glass (b–d) obtained in sets of tests with different plant species. Here, normalized data (divided by the corresponding value obtained in the first experiment on glass) are presented. ace, Acer negundo; alo, Aloe vera; aqu, Aquilegia vulgaris; bra, Brassica oleracea; chel, Chelidonium majus; chen, Chenopodium album; gl1, the first experiment on glass; gl2, the second experiment on glass; iri, Iris germanica; lac, Lactuca serriola; plant, waxy plant surface; tri, Trifolium montanum.

Table 1: Results of the paired $t$-test for comparisons between the first experiment on glass (gl1) and waxy plant surface (plant) and between the first (gl1) and second (gl2) experiments on glass for experimental sets with different plant species.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>$F_{\text{max}}$ gl1 vs plant</th>
<th>$F_{\text{max}}$ gl1 vs gl2</th>
<th>$F_{\text{peak1}}$ gl1 vs gl2</th>
<th>$T_{F_{\text{max}}}$ gl1 vs gl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acer negundo</td>
<td>$t = 10.821$ p = 0.001*</td>
<td>$t = 3.040$ p = 0.039*</td>
<td>$t = 5.305$ p = 0.006*</td>
<td>$t = 1.790$ p = 0.123</td>
</tr>
<tr>
<td>Aloe vera</td>
<td>$t = 15.193$ p = 0.001*</td>
<td>$t = 1.28$ p = 0.270</td>
<td>$t = 1.555$ p = 0.794</td>
<td>$t = 0.293$ p = 0.581</td>
</tr>
<tr>
<td>Aquilegia vulgaris</td>
<td>$t = 7.131$ p = 0.002*</td>
<td>$t = 1.087$ p = 0.338</td>
<td>$t = 0.048$ p = 0.964</td>
<td>$t = 0.037$ p = 0.331</td>
</tr>
<tr>
<td>Brassica oleracea</td>
<td>$t = 7.560$ p = 0.002*</td>
<td>$t = 2.790$ p = 0.049*</td>
<td>$t = 5.305$ p = 0.006*</td>
<td>$t = 1.951$ p = 0.123</td>
</tr>
<tr>
<td>Chelidonium majus</td>
<td>$t = 7.907$ p = 0.001*</td>
<td>$t = 1.215$ p = 0.291</td>
<td>$t = 1.975$ p = 0.119</td>
<td>$t = 0.385$ p = 0.720</td>
</tr>
<tr>
<td>Chenopodium album</td>
<td>$t = 10.206$ p = 0.001*</td>
<td>$t = 1.139$ p = 0.318</td>
<td>$t = 0.987$ p = 0.380</td>
<td>$t = 0.648$ p = 0.553</td>
</tr>
<tr>
<td>Iris germanica</td>
<td>$t = 10.746$ p = 0.001*</td>
<td>$t = 1.512$ p = 0.205</td>
<td>$t = 2.437$ p = 0.071</td>
<td>$t = 3.096$ p = 0.036*</td>
</tr>
<tr>
<td>Lactuca serriola</td>
<td>$t = 4.918$ p = 0.008*</td>
<td>$t = 2.041$ p = 0.111</td>
<td>$t = 3.490$ p = 0.025*</td>
<td>$t = 2.279$ p = 0.085</td>
</tr>
<tr>
<td>Trifolium montanum</td>
<td>$t = 10.088$ p = 0.001*</td>
<td>$t = 2.824$ p = 0.048*</td>
<td>$t = 1.818$ p = 0.143</td>
<td>$t = 1.539$ p = 0.199</td>
</tr>
</tbody>
</table>

*p, probability value; $t$, test statistics; *, significant difference.

The ability of C. fastuosa beetles, but the recovery time was relatively short [7]. Also, three other waxy plant surfaces studied here evoked a significant difference between the results of the first gl1 and the second gl2 experiments on glass, however, concerning only one of the attachment parameters measured: T. montanum regarding the maximum traction force $F_{\text{max}}$. 

**L. serriola** regarding the first peak of the traction force \(F_{\text{peak1}}\) and **L. germanica** regarding the time needed to reach the maximum traction force \(T_{\text{Fmax}}\). Since these plant surfaces are covered by middle-sized wax platelets with intermediate values of aspect ratio [34], they may yield a certain pad contamination, which in turn, may worsen the subsequent attachment ability of beetles for a short time. The waxy plant surfaces bearing small wax projections with low aspect ratio (especially compact, submicroscopic tubules in **A. vulgaris** and **C. majus**) caused inconsiderable pad contamination and, in turn, did not significantly affect further beetle attachment.

**Conclusion**

Traction experiments with tethered male individuals of the **Chrysolina fastuosa** beetles equipped with hairy adhesive pads clearly demonstrated a great reduction of attachment (maximum traction) force on all tested nine plant surfaces covered with three dimensional epicuticular waxes. The examination of adhesive pads after they had contacted the waxy plant substrates showed that (1) setal tips were contaminated by wax material and (2) the contamination degree differed between plant species depending on the micromorphology (primarily shape and size/aspect ratio) of the wax projections. The comparison of the maximum traction force value, the first peak of the traction force, and the time needed to reach the maximum force value in experiments on glass performed just before and immediately after the tests on the waxy plant surfaces revealed both significantly lower force values and significantly longer times in the case of the second experiment on glass compared to the first one in all tested insect individuals. When comparing the effect of different plant surfaces, this was more strongly pronounced in **A. negundo** and **B. oleracea** having wax projections with very high aspect ratios. These results evidently demonstrate that the impact of wax-covered plant surfaces on attachment to these surfaces and on subsequent attachment to a smooth surface is strongly influenced by the contamination of insect adhesive pads with the plant wax material.

**Experimental Plants**

Nine plants species from different plant families were used in the experiments: **A. negundo**, **Aloe vera** (L.) Webb. & Berth. (Asphodelaceae), **Aquilegia vulgaris** L. (Ranunculaceae), **Brassica oleracea** L. (Brassicaceae), **Chelidonium majus** L. (Papaveraceae), **Chenopodium album** L. (Chenopodiaceae), **Iris germanica** L. (Iridaceae), **Lactuca serriola** Torner (Asteraceae), and **Trifolium montanum** L. (Fabaceae). Young stems (**A. negundo**) or leaves (all other species) of these plants bearing 3D epicuticular wax coverage were collected near Jagotyn (Kyiv District, Ukraine; 50° 15’ 25” N, 31° 46’ 54” E) and used fresh in the force tests.

**Insect**

The leaf beetle **C. fastuosa** served as a model insect species in this study because it has been used in previous relevant experimental studies on insect attachment to various plant surface types [7] and contaminability of different plant waxes [34]. Additionally, it occurred in great numbers at the study site. The insects were used in the force experiments immediately after capture. In this study, only male beetles (body mass: 26 ± 6 mg, mean ± S.D., \(n = 10\)) were tested.

**Scanning electron microscopy**

To visualize the waxy plant surfaces and attachment devices in the **C. fastuosa** male beetle in both clean and contaminated conditions, scanning electron microscopy was employed. For plant surfaces, small (ca. 1 cm\(^2\)) pieces of plant organs were used. In the case of insect attachment organs, beetles were placed on a clean glass plate and their legs were cut off using a sharp razor blade. To get contaminated insect feet, a beetle was first allowed to walk on a fresh waxy plant surface for 1 min and then immediately transferred to the glass plate with the feet up, avoiding any contact, for cutting off the legs. Air-dried samples (parts of plant organs and clean or contaminated insect legs) were mounted on holders, sputter-coated with gold–palladium (thickness 8 nm for plants and 10 nm for insects), and examined in a Hitachi S-800 scanning electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) at an acceleration voltage of 2–20 kV (plants) or 20 kV (insects). In the characterization of the waxy plant surfaces, we used the classification of plant epicuticular waxes according to [45].

**Force measurements**

Force experiments were carried out using a load cell force sensor FORT-10 (10 g capacity; World Precision Instruments Inc., Sarasota, FL, USA) connected to a force transducer MP 100 (Biopac Systems Ltd., Santa Barbara, CA, USA) [24,46]. First, in order to make a test beetle incapable of flying, its elytra were glued together with a small drop of molten beeswax. At the same time, a 10–15 cm long human hair was stuck to the wax drop. After the wax had hardened and the insect recovered from the treatment, a free end of the hair was attached to the force sensor. Then, the tethered beetle walked on a horizontally placed test substrate pulling the hair for ca. 30 s, while the friction (traction) force thus produced by the moving insect was registered. Since the insects walked parallel to the measurement axis of the sensor, the recorded force corresponded to the total traction force. Force–time curves obtained were used to estimate the maximal traction force \(F_{\text{max}}\), the value of the first peak of the traction force \(F_{\text{peak1}}\), and the time \(T_{\text{Fmax}}\) needed to reach the maximum traction force value (Figure 5a).
With each insect individual, three successive force tests were carried out on the following substrates: (1) a smooth hydrophilic glass used as a reference substrate (g1), (2) a waxy plant surface (plant), and (3) once more a glass surface for comparison (g2). Taking into consideration that these waxy plant surfaces are capable of contaminating insect attachment organs with wax particles [34], we performed the second experiment on glass immediately after the test on the plant, in order to completely exclude a possible effect of feet cleaning or grooming by insects. This aided in the examination of the influence of dirty adhesive pads on the subsequent attachment ability of the beetles. On each set of substrates, five individual male beetles were tested. In all, 135 force experiments were conducted. Force tests were carried out at 22–25 °C temperature and 60%–75% relative humidity.

The statistical analyses of the values of the maximum traction force \( F_{\text{max}} \), the first peak of the traction force \( F_{\text{peak1}} \), and the time \( T_{\text{max}} \) needed to reach the maximum traction force for the comparisons between g1 and plant and between g1 and g2 were performed using the paired t-test (SigmaStat 3.5, Systat Software Inc., Point Richmond, CA, USA). The comparisons were conducted for both (1) data on all test insects pooled together, that is, experiments with all waxy plant surfaces (d.f. = 44) and (2) data obtained from five test insects on each plant surface separately (d.f. = 4).

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Data Availability Statement
The data that supports the findings of this study is available from the corresponding author upon reasonable request.

References

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Comparative analysis of the ultrastructure and adhesive secretion pathways of different smooth attachment pads of the stick insect *Medauroidea extradentata* (Phasmatodea)

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### Full Research Paper

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### Abstract

The mechanism by which insects achieve attachment and locomotion across diverse substrates has long intrigued scientists, prompting extensive research on the functional morphology of attachment pads. In stick insects, attachment and locomotion are facilitated by two distinct types of smooth cuticular attachment pads: the primary adhesion force-generating arolium and the friction force-generating euplantulae. They are both supported by an adhesive secretion delivered into the interspace between the attachment pads and the substrate. In this study, we analysed and compared internal morphology, material composition and ultrastructure, as well as the transportation pathways in both adhesive organs in the stick insect *Medauroidea extradentata* using scanning electron microscopy, micro-computed tomography, light microscopy, and confocal laser scanning microscopy. Our observations revealed structural differences between both attachment pads, reflecting their distinct functionality. Furthermore, our results delineate a potential pathway for adhesive secretions, originating from exocrine epidermal cells and traversing various layers before reaching the surface. Within the attachment pad, the fluid may influence the viscoelastic properties of the pad and control the attachment/detachment process. Understanding the material composition of attachment pads and the distribution process of the adhesive secretion can potentially aid in the development of more effective artificial attachment systems.

### Introduction

Throughout their evolutionary timeline, insects evolved various surfaces interacting with the environment. These include friction-based adhesive organs, which are essential for locomotion by generating frictional and adhesive forces [1-4]. Two morphologically different friction-based adhesive principles convergently emerged in insects multiple times: hairy and smooth...
adhesive organs [5-7]. Both principles are used for multiple functions from locomotion [8,9] to attachment during copulation [10] and predator resistance [11].

To fulfil their functions, smooth attachment pads need to enhance the actual contact area between the pad and the substrate for the realisation of efficient attachment due to adhesion and friction forces [3,9,12-14]. Smooth attachment pads have independently evolved in most large insect groups, possessing multiple specialized types of pads on the same leg that are adapted to attachment through the division of labour by preferably generating more adhesion or friction [5]. Adhesive secretion in the contact zone between the attachment pad and substrate supports the functionality of the pads [15].

The adhesive secretion can fill the gaps in the substrate roughness and thereby increase the contact area [14,16-19]. It can aid in the enhancement of viscous and capillary forces further increasing the attachment strength [9,14,20-24]. The adhesive secretion can be essential for the self-cleaning mechanism by binding smaller contamination particles together into larger complexes for easier removal [25,26]. It can also improve attachment to surfaces with different surface chemistry by mediating between the two surfaces in contact [27,28]. The lipid-containing pad secretion protects the insect from additional water loss through the thin-walled attachment pads [29] and assists in chemical communication [30].

The tarsal secretion can facilitate these functions due to its chemical composition and the resulting physical properties. Chemical analyses of the tarsal fluid revealed that its composition differs between different insect groups but mostly contains water-soluble and lipid-soluble substances [31-35] creating lipid droplets in an aqueous fluid [27,36] or hydrophilic nanodroplets embedded in an oily continuous phase [23,37]. Additionally, the tarsal secretion could be a mixture of multiple substances that are present in varying mixture ratios, which would also influence its properties and thus its functions [38]. Secretion with more long-chain carbons and higher branching bonds is more viscous and would potentially exert stronger viscous forces [39,40].

The functional differentiation of the smooth attachment pads likely arises from differences in the ultrastructure and material composition of the pad types and is potentially supported by possible differences in the produced tarsal secretion. Despite extensive research on the attachment capabilities and the ultrastructure of the different attachment pads in various insect groups (for example, Coleoptera [5], Hemiptera [41], Diptera [42,43], Orthoptera [5,20,44], and Blattodea [45]), knowledge on the differences in the internal ultrastructure and fluid transportation between different types of smooth attachment pads located on the same tarsus is scarce, especially in Phasmatoidea. Recent investigations of the ultrastructure and material properties of the smooth tarsal attachment pads of phasmsids complement our information on the morphology of the droplets [38], biomechanics of their attachment performance [28,46-51], and the complementarity of the two pad types [47,52,53].

In this study, we compare the ultrastructure and material composition of the two smooth tarsal (euplantulae) and pretarsal (arolium) attachment pads of the stick insect Medauroidea extradentata (Brunner von Wattenwyl, 1907), focusing on their functional differences as well as on the tarsal secretion production pathways. It was previously shown that the euplantulae are used to generate stationary attachment forces and propulsion (frictional pad) and the arolium to generate adhesion forces (adhesion pad) [52,54]. M. extradentata was selected here due to its relatively large adhesives organs that bear no further surface microstructures [47,55,56] and because the droplet morphology of its tarsal secretion has been recently analysed [28,38,47,55,56].

Combining different imaging techniques, including scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM), histological staining of longitudinal and cross sections (toluidine blue and Cason), and micro-computed tomography (µCT), our investigation of the arolium and euplantulae of the stick insect M. extradentata addresses the following questions: (1) Are there structural and material differences between the tarsal frictional pads (euplantulae) and the pretarsal adhesion pads (arolia)? (2) Where is the adhesive secretion produced and stored? (3) How many different types of exocrine cells producing pad secretions do exist? (4) How is the adhesive secretion transported from the production site to the pad surface? The results could enhance our overall comprehension of the functionality of the two smooth attachment organs, euplantulae and arolium, also shedding light on the fluid production and transportation processes in different smooth pads of Phasmida.

Materials and Methods

Animal

We used the phasmid species Medauroidea extradentata (Brunner von Wattenwyl, 1907) (Figure 1A), because of the availability of livestock and the presence of the functional morphology data on its tarsal attachment system [28,46-49].

Individuals were obtained from the laboratory cultures of the Department of Functional Morphology and Biomechanics (Kiel University, Germany). The insects were fed with blackberry leaves ad libitum and kept in a regular day and night cycle.
Only adult female individuals were selected. The animals were kept with blackberry leaves in clean hard plastic boxes to reduce contamination of the attachment pads.

**Light microscopy**

Two tarsi of adult female *M. extradentata* were dissected into five tarsomers. The proximal four tarsomers bear one euplantula each, whereas the fifth tarsomere additionally carries the pretarsus including the arolium (see Figure 1B, Figure 1C). The five tarsomers were fixed in 2.5% glutaraldehyde in (pH 7.4) phosphate-buffered saline (PBS) for 24 h, washed two times in PBS for 30 min each, fixed in 1% aqueous OsO$_4$ for 1 h, and washed two times in double-distilled water, for 30 min each. After fixation, the samples were dehydrated using an ascending ethanol series from 30% to 100% (each step for 20 min). All steps were performed on a shaker and at 4 °C. For the last step, the samples were embedded in Epon 812 (Glycidether 100; Carl Roth GmbH, Karlsruhe, Germany) and polymerized at 60 °C for 48 h.

The embedded samples were cut into semi-thin sections of 0.2–1.0 µm using a Leica EM UC7 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) at 21.5 °C room temperature, mounted on polylysine-covered glass slides (Gerhard Menzel GmbH, Braunschweig, Germany) and stained with toluidine blue or Cason’s triple stain (Romeis 2010). Toluidine blue is a basic metachromatic dye, which selectively stains basophilic tissue components and has a high affinity to acidic tissue (nucleic acids are stained blue and polysaccharides purple). Previous experiments have also shown that the dye stains soft parts of the cuticle dark blue, and sclerotized parts of the cuticle light blue. In addition, the blue colour intensity corresponds to the relative electron density of the tissue in TEM [57–59].

Cason’s triple stain allows for the differentiation of differently sclerotized regions from brown over orange to yellow (with a decreasing degree of sclerotization) to resilin-bearing regions stained from violet to pink [60,61].

For staining with toluidine blue, the glass slides were incubated with 0.1% toluidine blue solution for 2 min and rinsed using a stream of distilled water. Cason’s triple stain (consisted of 1 g of phosphotungstic acid, 2 g of orange G, 1 g of aniline blue, and 3 g of acid fuchsin, dissolved in 200 mL of distilled water [60,61]). Cason stain was applied onto the glass slides for 5 min at 60 °C and rinsed with 70%–100% EtOH and tap water.

The stained samples were observed using a light microscope (Zeiss Axioplan, Carl Zeiss Microscopy GmbH, Jena, Germany) with 40× and 100× lenses. The images were processed using Adobe Photoshop (version CS6; Adobe Systems Inc., San Jose, CA, USA).

**Scanning electron microscopy**

Tarsi of *M. extradentata* were cut from adult females and fixed in 2.5% glutaraldehyde in PBS for 24 h. Then, they were washed two times with PBS for 30 min and two times with double-distilled water for 30 min each. Afterwards, the samples were dehydrated in an ascending ethanol series. Each step was performed on ice (4 °C) and on a shaker. Afterwards, the samples were critical point dried (Leica EM CPD300, Leica, Wetzlar, Germany). Then, the dry pretarsal arolium and tarsal euplantulae were dissected at the centre using two fine tweezers to achieve a clean breaking edge. The samples were mounted on aluminium stubs and sputter-coated with a 10 nm layer of gold–palladium (Leica BalTec SCD 500, Leica, Wetzlar, Germany). The images were obtained using a scanning electron microscope (TM 3000, Hitachi High-Technologies Corp, Tokyo, Japan) at 3 kV acceleration voltage. The recorded images were stitched, merged, and processed using the software Photoshop CS6 (Adobe Systems Inc., San Jose, CA, USA).

**Confocal laser scanning microscopy**

Whole tarsi and cross sections of the pretarsal (arolium) and tarsal (euplantulae) attachment pads of adult female stick insects *M. extradentata* were analysed using CLSM. Fresh tarsi of *M. extradentata* were cut off, directly placed in 100% Triton X-100 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 30 min, and then transferred to glycencine. To analyse the entire tarsus, it was directly transferred onto a glass slide and mounted with a coverslip (thickness = 0.170 ± 0.005 mm, refractive index = 1.52550 ± 0.00015, Carl Zeiss Microscopy GmbH, Jena, Germany). For the cross sections of arolium and euplantulae, the attachment pads were cut with a carbon blade and individually transferred onto a glass slide and mounted with a coverslip (specifications as above).

For analysis, a confocal laser scanning microscope (Zeiss LSM 700, Carl Zeiss Microscopy GmbH, Jena, Germany) and four stable solid-state lasers (wavelengths 405, 488, 555, and 639 nm in combination with the respective bandpass and longpass emission filters BP420–480, LP490, LP560, LP640 nm) were used.

The whole tarsi were visualised with a 5× lens (Zeiss Plan-Apochromat, air immersion, numerical aperture = 0.16, Carl Zeiss Microscopy GmbH, Jena, Germany) and the cross sections of the attachment pads with a 20× lens (Zeiss Plan-Apochromat, air immersion, numerical aperture = 0.17, Carl Zeiss Microscopy GmbH, Jena, Germany). Maximum intensity projections were created using the ZEN 2008 software.
(https://www.zeiss.de/mikroskopie) and subsequently, the contrast and brightness were adjusted in Adobe Photoshop (version CS6; Adobe Systems Inc., San Jose, CA, USA). Three colours: red, green, and blue were assigned according to the emitted autofluorescence wavelength representing to some extent the degree of sclerotization. Red represents the highest sclerotization degree, green – the medium one, and blue – the lowest one (see Figure 1B).

Micro-computed tomography
A whole tarsus of an adult female *M. extradentata* was cut off at the base of the tibia, directly fixed in 2.5% glutaraldehyde in PBS, and washed in PBS. For the preparation of the µCT scan, the tarsus was dehydrated with an ascending EtOH sequence at 4 °C on a shaker, and subsequently critical point dried using Leica EM CPD300 (Leica, Wetzlar, Germany). The tarsus was scanned using a Skyscan®1172 µCT (Bruker micro-CT; CT-scanner settings: X-ray source: 40 kV, 250 μA, 360 rotation, 0.2 rotation step, 10 frames averaging, and 10 random movements), reconstructed in Nrecon®1.0.7.4 (Bruker micro-CT, Billerica, MA, USA), segmented with Amira®6.2 (Thermo Fisher Scientific, Waltham, MA, USA), and visualized with the open-source 3D creation software Blender 2.82a (Blender Foundation, Amsterdam, Netherlands) and Affinity Designer (Serif, Nottingham, UK).

Results
Tarsal structure
The structure of the tarsus of *M. extradentata* was observed using CLSM and SEM (Figure 1B,C). It comprises five tarsomeres (ta 1–5) and the pretarsus. Tarsomeres one to four (ta 1–4) each bear a pair of euplantulae (eu 1–4) at their distal ends. The pretarsus features the arolium (ar) situated between a pair of claws (cl). The euplantulae, the cuticle between them,
and the arolium bear a rather smooth surface structure. The remaining surface of the tarsomeres, where no attachment pads are situated, is covered with setae (Figure 1C). The CLSM images revealed that both types of attachment pads and the cuticle between the euplantulae and between the tarsomeres show a low degree of sclerotization (blue coloration). In contrast, the cuticle of the remaining tarsomeres has a higher degree of sclerotization (green/yellow coloration). Notably, the distal ventral region of the arolium displays a relatively higher degree of sclerotization (green/yellow coloration). Additionally, red coloration is visible inside the arolium; however, this does not correspond to the cuticle, but presumably to the glandular tissue of the arolium (Figure 1B).

Arolium structure

The pretarsus of *M. extradentata* is 500 µm wide and 400 µm long. The ventral face of the arolium consists of a thickened layer of fibrous cuticle composing the actual smooth attachment pad (ap) [1]. Toluidine blue staining resulted in a blue hue of the attachment pad, indicating the presence of a meshed network of flexible cuticle fibres within the attachment pad (Figure 2B). This coarse meshed-fibre structure was also observed in SEM (Figure 2C). In addition, using CLSM, the attachment pad structure exhibited a low degree of sclerotization indicating a presumably soft cuticle (Figure 2D). Internally, the main part of the arolium consists of a large epithelium, recognizable by the light hue of the toluidine blue staining. The epithelium mainly consists of exocrine cells (ex) which display a large surface area towards the hemolymph due to irregular protrusions (Figure 2B). These evaginations are also visible in the µCT cross sections as radio-dense layers (Figure 2A). The exocrine cells exhibited a mixed red/blue signal in CLSM (Figure 2D) and appeared densely packed in the SEM sections (Figure 2C). The exocrine cells are likely surrounded by the hemolymph (he), which appeared yellowish when stained with toluidine blue (Figure 2A). On the back of the arolium, epidermal cells (ep) are present, separated from the exocrine cells by the hemolymph (Figure 2B). These epidermal cells were stained in a relatively darker hue by toluidine blue (Figure 2B) and displayed a reddish fluorescence signal in CLSM (Figure 2D). However, they were not visible in the µCT cross sections (Figure 2A).

The arolium exhibits a sclerotized cuticle (cu) on its dorsal side. The sclerotized cuticle is composed of two layers, the inner...
layer showing light blue staining by toluidine blue (Figure 2B) and a light red fluorescence signal in CLSM (Figure 2D), while the outer layer is stained dark blue by toluidine blue (Figure 2B) and shows a dark red fluorescence signal in CLSM (Figure 2D). Both layers show radiodensity in µCT (Figure 2A).

**Arolium ultrastructure**

The internally located ≈10 µm wide endocuticle layer 1 (e1) is characterized by its loose, parallel arrangement of sheets, which are discernible through their red staining with Cason (Figure 3A) and blue staining with toluidine blue (Figure 3B). This parallel arrangement is also evident in SEM (Figure 3D) and in longitudinal microtome sections in the light microscope (Figure 3B). In CLSM, the endocuticle layer 1 exhibits a relatively low degree of sclerotization (Figure 3C).

On top of the endocuticle layer 1 there is a ≈30 µm thick primary rod layer (pr) consisting of wide rods extending towards the surface of the arolium and branching into finer rods forming another ≈10 µm thick branching rod layer (br) (Figure 3). The primary rod layer and the branching rod layer are notably stained red by Cason stain (Figure 3A) and blue by toluidine blue stain (Figure 3B), confirming their cuticular origin. The CLSM images further revealed that both layers emit a blue signal, indicative of the presence of resin (rubber-like protein) with relatively soft properties (Figure 3C). The morphological details of these layers are also apparent in longitudinal microtome sections (Figure 3B) and SEM sections (Figure 3D). The primary rod layer is comprised of relatively thick cuticle fibres that branch into finer ones within the branching rod layer, terminating in the superficial layer (sf) (Figure 3D).

The superficial layer is the outermost layer in the arolium and is in direct contact with the environment. When examined with a light microscope, this layer appeared remarkably smooth. Notably, Cason staining resulted in a deep red hue, while toluidine blue staining resulted in a dark blue coloration (Figure 3A, Figure 3B), indicating that the superficial layer consists of a more densely packed cuticle if compared to the rods of the primary rod layer and branching rod layer. Additionally, the cuticle of the superficial layer displays a low degree of sclerotization as indicated by CLSM results (Figure 3C).

**Arolium exocrine cells**

The exocrine cells (ex) of the epidermal cell layer are separated from the hemolymph reservoir (he) by a basal layer (bl) which is stained light blue by toluidine blue. (Figure 3B, Figure 3E). The identification of exocrine cell bodies is facilitated by their blue coloration when stained with toluidine blue (Figure 3B, Figure 3E), alongside the presence of a thick basal lamina and numerous discernible cellular structures. When observed in CLSM, the exocrine cells exhibit a red autofluorescence signal (Figure 3C). Notably, the exocrine cells possess large nuclei (nu) with multiple nucleoli, which are prominently stained in shades of blue by toluidine blue (Figure 3B, Figure 3E). Light microscopy revealed the presence of numerous vesicles (ve), which can be distinguished as either black when stained with toluidine blue and Cason or show an orange colour without staining (Figure 3A,B,E). When observed using SEM, these vesicles appear smooth and appear to be detached from the surrounding cellular structures (Figure 3F). Furthermore, round and unstained areas were observed (Figure 3A,B,E). When examined in SEM, these structures appear as hollow, empty spaces (Figure 3F). These structures are named hollow spaces (hs). Based on all these characteristics, the exocrine cells of the epidermal cell layer are likely classified as exocrine cells type I [62].

The basal and apical sides of the exocrine cells exhibit surface expansions towards the basal layer (basal) and the adhesive secretion reservoir (as) (apical) (Figure 3B). The adhesive secretion reservoir is stained light blue with toluidine blue and is situated between the exocrine cells and the epicuticle layer 1 (Figure 2A; Figure 3B).

**Tarsomere structure**

Only tarsomeres that possess an attachment pad (euplantulae) were examined and are described below. These tarsomeres measure ≈330 µm in length and ≈210 µm in width (depending on the tarsomere).

The septa (se) separate the interior of the tarsomere into four sections. Two thin septa laterally segregate it into two areas on the ventral side (vn), while a comparably thicker septa separates the tarsomere into central and dorsal areas. The central area (ca) accommodates the tendon (te), and the dorsal area (da) the tracheal structures (tr) and nerve bundles (nb). Notably, each of these areas possesses an individual hemolymph channel for circulatory and possible structural purposes through hydrostatic pressure (Figure 4). The septa are dyed blue by toluidine blue and show a parallel cuticle layering in SEM (Figure 4B, Figure 4C).

The cuticle on the ventral part of the dorsal area shows distinctive morphological and structural characteristics compared to the rest of the cuticle, as it lacks the typical toluidine blue staining and autofluorescence of the sclerotized cuticle. In contrast, the region is stained light blue with toluidine blue and exhibits a low degree of sclerotization in CLSM. Moreover, it presents a unique morphology, appearing fanned out, suggesting a more flexible structure (Figure 4B, Figure 4D). Based on these characteristics, this cuticle region is named flex-
Figure 3: Arolium material structure visualised using different techniques. Detailed images of the adhesive pad of the arolium. The different methods highlight the morphological and structural characteristics of the respective layers and structures. (A) Light microscopy image of the cross section stained with the Cason triple stain. (B) Light microscopy image of the longitudinal section stained with toluidine blue. (C) CLSM image of the cross section. (D) SEM image of the cross section. (E) Light microscopy of the longitudinal section of the exocrine cells stained with toluidine blue. (F) SEM image of the cross section of the exocrine cells. The ventral side of the arolium is oriented towards the bottom of the pictures. As = adhesive secretion reservoir; bl = basal layer; br = branching rod layer; e1 = endocuticle layer 1; ex = exocrine cells; he = hemolymph; hs = hollow spaces; nu = nucleus; pr = primary rod layer; sf = superficial layer; ve = vesicles.
Figure 4: Morphology of the tarsomere. The internal ultrastructure of the tarsomere was visualized using four different methods, which show the different layers and highlight their morphological and structural characteristics. The following methods were used: (A) µCT image of the cross section. (B) Light microscopy cross section stained with toluidine blue. (C) SEM overview of the entire tarsomere. (D) CLSM cross section of the tarsomeres. The ventral sides of the euplantulae are oriented towards the bottom of the images. The examined sections originate from individual tarsomeres along the tarsus, whereby the length and width proportions can differ. ap = attachment pad; ca = central area; cp = connective pad; cu = sclerotized cuticle; da = dorsal area; ex = exocrine cells; fc = flexible cuticle; ha = hair/seta; he = hemolymph; nb = nerve bundle; se = septum; te = tendon; tr = trachea; vn = ventral area.

The µCT imaging of the ventral side of the euplantulae revealed a dense hull (lighter grey) and a more X-ray transparent body (darker grey) (Figure 4A). Toluidine blue staining detected a darker blue stained hull and a lighter blue body (Figure 4B). The SEM images unveiled a rather smooth surface topography (Figure 4C). Furthermore, CLSM detected a weak degree of sclerotization (blue autofluorescence signal) of the whole structure (Figure 4D). All these features indicate that this ventral structure is the euplantula attachment pad (ap) that makes direct contact with the substrate. The attachment pad is ≈60 µm wide and laterally merges with the sclerotized cuticle of the tarsomere. This is recognizable by the different coloration of the lateral exoskeleton which shows the staining by toluidine blue and CLSM autofluorescence wavelength signals typical for the sclerotized cuticle (Figure 4B, Figure 4D). The attachment pads of the tarsomeres internally extend into the corresponding tarsomere.

The structure connecting the two attachment pads shows morphological similarities with the attachment pad. In the µCT, the outer hull of this structure exhibits high radiodensity and the inner body shows lesser density (Figure 4A). Similarly, light microscopy with toluidine blue staining showed the outer hull in dark blue and the inner body in a lighter shade of blue (Figure 4B). The SEM images revealed a smooth surface (Figure 4C), while CLSM analysis demonstrated a low degree of sclerotization, suggesting the presence of soft cuticle (Figure 4D). Due to these morphological similarities and the fact that this structure connects the attachment pads, it is referred to as a connective pad (cp).

On the internal side of both the attachment pad and connective pad, an epidermal cell layer is situated. This layer encompasses the entire surface of the ventral interior of the tarsomeres, restricting the hemolymph reservoir inside. The layer is separat-
ed from the remaining tarsomere tissue by septa. The epidermal cells appear radiolucent in the µCT cross sections (Figure 4A) and are stained blue with toluidine blue (Figure 4B). Also, they show a weak green autofluorescence signal in CLSM (Figure 4D). These findings indicate that the epidermal cell layer consists of exocrine cells (ex). Furthermore, the lateral sides of the tarsomeres exhibited discernible nerve bundles and hair/seta attachment sites (ha), extending into the epidermal layer (Figure 4B).

**Euplantulae ultrastructure**

The inner layer of the attachment pad (ap) is ≈1.5 µm wide, stained light red and blue by Cason and toluidine blue, respectively (Figure 5A, Figure 5B), exhibiting a low degree of sclerotization in CLSM (Figure 5C) and composed of parallel layers of cuticle sheets (Figure 5D). This composition identifies the layer as the endocuticle layer 1 (e1).

From the endocuticle layer 1 emerges a ≈12 µm thick layer of dense wide rods, which subsequently ventrally branches towards the surface into finer, denser rods, and finally terminate into a ≈4 µm thick superficial layer (sf) (Figure 5A,B,D). The layer composed of thick rods is the primary rod layer (pr) and the layer with the finer rods is the branching rod layer (br) (Figure 5D). Cason and toluidine blue staining resulted in a lighter red and blue coloration, respectively, for the cuticle of the primary rod layer compared to that of the branching rod layer, likely reflecting the denser fibrous structure of the latter (Figure 5A, Figure 5B). The CLSM analysis revealed a low degree of sclerotization in both layers, suggesting soft cuticle, with discernible regions of reddish autofluorescence signals, possibly attributed to residual adhesive secretions within the cuticle layers, or to underlying epidermal cells (Figure 5C).

The finer fibers of the branching rod layer ultimately terminate in the superficial layer (Figure 5A,B,D). The thin superficial layer is the outermost layer of the euplantulae, establishing direct contact with the substrate (Figure 5D). Examination in the light microscope and SEM revealed a smooth surface of the pad (e.g., Figure 4C). Staining with Cason and toluidine blue resulted in a dark red or dark blue hue, respectively, indicative of a tightly packed cuticle (Figure 5A, Figure 5B). Additionally, CLSM unveiled a low degree of sclerotization in the superficial layer (Figure 5C).

**Euplantulae exocrine cells**

The hemolymph reservoir (he) is ventrally surrounded by a layer of epidermal cells. The basal region of this layer establishes direct contact with the hemolymph with evaginations increasing the contact surface area (Figure 5B). When stained with toluidine blue or Cason, the epidermal cell layer displays deep blue and light red colorations, respectively (Figure 5A,B,E). In CLSM, the layer exhibited a strong green signal with weak red signal portions (Figure 5C). The cells within the epidermal layer house a prominent nucleus with multiple nucleoli, stained in a deeper blue and red by the two staining methods, respectively (Figure 5A,B,E). Due to these characteristics, the cells within the epidermal layer are identified as exocrine cells (ex). Additionally, light microscopy images revealed vesicles (ve) inside the cells. These either exhibited substantial staining intensity due to the applied staining methods or displayed an orange coloration without staining (Figure 5A,B,E). Upon examination through SEM, they appeared spherical and presented either a smooth or slightly rough surface (Figure 5F). Within the exocrine cell layer, unstained larger hollow spaces (hs) were observed (Figure 5A,B,E). Examination via SEM revealed these hollow spaces to appear within the exocrine cell layer, after chemical fixation and critical point drying (Figure 5F). These morphological characteristics identify these cells within the euplantulae as exocrine cells type I [62]. The exocrine cells are enveloped and separated on the ventral side by a thin cuticle layer. This layer is notably more intensely stained in blue (toluidine blue) and red (Cason) compared to the exocrine cells, and is identified as the endocuticle layer 2 (Figure 5A, Figure 5B). The endocuticle layer 2 ventrally borders a ≈2 µm wide layer which runs along the entire length of the attachment pad and the connective pad, laterally terminating into the sclerotized cuticle of the tarsomeres. This layer is very lightly stained by toluidine blue and Cason (Figure 5A, Figure 5B) and named adhesive secretion reservoir (as).

**Connective pad**

The connective pad medially connects the two euplantulae (Figure 4A, Figure 4B; Figure 6A, Figure 6B). The ultrastructure of the connective pad comprises two layers of parallel cuticle sheets with a ventral terminating superficial layer (sf). The adhesive secretion reservoir and exocrine cells of the euplantulae internally extend and connect the tissues of the two euplantulae (Figure 5). The two parallel cuticular layers are distinguishable in terms of coloration through Cason and toluidine blue staining. The layer situated dorsally adjacent to the adhesive secretion reservoir, exhibited a light red hue stained with Cason and a light blue hue with toluidine blue, identifying it as the endocuticle layer 1 (e1). The outer layer presented a more intense coloration identifying it as the outer parallel layer (op) (Figure 6A, Figure 6B). The CLSM analysis indicates a blue indistinguishable autofluorescence signal in both layers, indicating their low degree of sclerotization (Figure 6C). The SEM images revealed structural similarities between the two layers, with the outer parallel layer displaying a slightly denser layering (Figure 6D). The morphology of the superficial layer in
Figure 5: The euplantula sections. Detailed images of the attachment pad of the euplantula. The different methods highlight the morphological and structural characteristics of the respective layers and structures. (A) Cross section stained with Cason’s stain, light microscopy. (B) Longitudinal section stained with toluidine blue, light microscopy. (C) Cross section in CLSM. (D) Cross section in SEM. (E) Longitudinal section of the exocrine cells stained with toluidine blue, light microscopy. (F) Cross section of the exocrine cells in SEM. The ventral side of the euplantulae is oriented towards the bottom of the images. as = adhesive secretion reservoir; br = branching rod layer; e1 = endocuticle layer 1; e2 = endocuticle layer 2; ex = exocrine cells; he = hemolymph; hs = hollow spaces; nu = nucleus; pr = primary rod layer; sf = superficial layer; ve = vesicles.
Figure 6: The connective pad between neighbouring euplantulae. Detailed images of the connective pad. The different methods highlight the morphological and structural characteristics of the respective layers and structures. (A) Cross section of the connective pad stained with Cason’s stain, light microscopy. (B) Longitudinal section of the connective pad was stained with toluidine blue, light microscopy. (C) Cross section in the CLSM. (D) Cross section in the SEM. The ventral sides of the connective pads are oriented towards the bottom of the images. as = adhesive secretion reservoir; e1 = endocuticle layer 1; ex = exocrine cells; op = outer parallel layer; pl = parallel layer; sf = superficial layer.
the connective pad corresponds to the characteristics of the superficial layer in the attachment pads, exhibiting a more intense staining with Cason and toluidine blue (Figure 6A, Figure 6B), a low degree of sclerotization (Figure 6C), and a dense cuticle organization, evident via SEM, than that of the outer parallel layer and endocuticle layer 1 (Figure 6D). Both the exocrine cells and the adhesive secretion reservoir of the connective pad exhibit the same morphological characteristics as those of the attachment pads (Figure 5; Figure 6).

Additional morphological observations
The superficial layer of the connective pad bears distinctive spherical shapes, which are situated on the dorsal ridges of the connective pad in proximity to the central region of the tarsomere (Figure 7A, Figure 7B). These putatively anti-adhesive structures (aa) were also discovered on the dorsal edge of the arolium (Figure 3B). The SEM and light microscopy (toluidine blue staining) images revealed pore openings (po) in the superficial layer of the euplantulae (Figure 7C, Figure 7E). In addition, small spherical bodies were observed throughout the primary rod layer and branching rod layer, as well as directly beneath the superficial layer of the euplantulae and were identified as adhesive fluid residues (as) (Figure 7C, Figure 7D).

Discussion
Similarities between the two attachment pad types
The anatomy and material composition of the two tarsal attachment organs, euplantulae and arolium, were compared using different imaging techniques. The study revealed some similarities between them, corresponding to their roles in the attachment process [1]. In the interior of both organs, there is a hemolymph reservoir serving dual purposes as a hydrostatic support system and a supply of nutrients to the cells [63]. Following the hemolymph reservoir, exocrine cells are present in the epidermal layer of both organs. As transformed epidermal cells, the exocrine cells are responsible for the secretion of all cuticular layers apical to them, as well as the production of the adhesive secretion. These layers encompass the endocuticle layers 1 and 2, the adhesive secretion reservoir, the primary and branching rod layers, as well as the superficial layer [41,64]. These exocrine cells exhibit surface extensions into the hemolymph and adhesive secretion reservoir optimizing the substance absorption and discharge [31,65-67]. Adjacent to the exocrine cells is the adhesive secretion reservoir serving for the accumulation of the produced adhesive secretion. Both pad types share a similar organisation of the procuticle. The endocuticle layer 2 has a parallel cuticle layering, the primary rod layer is composed of wide cuticle rods ventrally branching into finer rods within the branching rod layer, terminating in the superficial layer (Figure 3; Figure 5).

Previous investigations of the smooth attachment pads (arolium and euplantulae) of Gromphadorhina portentosa (Schaum, 1853) by Schmitt and Betz [45] revealed a similar layering of both attachment pads. Similar structures of the procuticle, especially the primary rod-, branching rod-, and superficial layer were also reported by Gorb et al. [20], Gorb and Scherge [21], and Goodwyn et al. [44] in the smooth euplantulae of Tetigonia viridissima (L., 1758) and Locusta migratoria (L., 1758). Differences in the layering and the details of microstructure likely evolved due to variations in their ecological lifestyle.

Several insects possess hairy attachment organs, which morphologically differ from the smooth ones examined herein. The differences between them manifest primarily in the morphology of the procuticle region. Hairy attachment organs are characterized by cuticle outgrowths (e.g., setae or acanthae [5,68-71]), whereas smooth attachment organs consist of hierarchically split cuticle rods terminating in the superficial layer creating a rather smooth surface [20,70,71]. Both types of attachment organs utilise their distinct morphologies to efficiently replicate the substrate profile to a similar extent, thereby amplifying the actual contact area and, consequently, enhancing attachment [1,2,72,73].

Differences between smooth and hairy attachment pads
The primary difference between hairy and smooth attachment organs manifests in the cuticular morphology. Hairy attachment organs consist of cuticle outgrowths (e.g., setae or acanthae [5,68-70]), the cuticle of smooth attachment organs consists of filaments that hierarchically split terminating in the superficial layer, creating a rather smooth surface at the level of light microscopy [20,70,71].

Both types of attachment pads efficiently replicate the surface profile of the substrate owing to their distinct structures, thereby augmenting the actual contact area and, consequently, enhancing attachment. Smooth attachment pads accomplish this through hierarchical organization and the viscoelastic properties of the cuticle [1,2,72,73].

Differences between the two attachment pad types
Despite the similar overall morphology, the two attachment organs show some distinct structural differences, which can be attributed to different functions that both types fulfill. Previous research on the attachment pads of the phasmid Carausius
Figure 7: Detailed images of additional morphological observations. The different methods highlight the morphological and structural characteristics of the respective layers and structures. (A) The top view on the euplantulae of one tarsomere shows the connective pad, SEM. (B) Longitudinal section of the connective pad was stained with toluidine blue, light microscopy. (C, D) Cross sections of the arolium, SEM. (E) Superficial layer, SEM. (D) Primary rod layer and branching rod layer, SEM. (E) Longitudinal section of the euplantula stained with toluidine blue, light microscopy. (F) Cross section of the euplantula stained with toluidine blue, light microscopy. aa = anti-adhesive structures; af = adhesive fluid; br = branching rod layer; cp = connective pad; he = hemolymph; po = pore opening; pr = primary rod layer; se = septum; te = tendon.

*morosus* (Brunner von Wattenwyl, 1907) and the cockroach *Nauphoeta cinerea* (Olivier, 1789) proposed that the arolium primarily serves to generate adhesion, while the euplantulae predominantly function for the generation of friction, characterizing the arolium as an adhesive pad and the euplantulae as friction pads [52-54]. Adaptation to the specific requirements is realized in euplantulae and arolia by the different morphological organizations.
Primary rod layer and branching rod layer
In the arolium, the fibres in the primary rod layer and branching rod layer are notably thicker and more widely spaced compared to those in the euplantulae (Figure 3 (arolium); Figure 5 (euplantulae)). In general, the hierarchical organization of the fibres enables local deformation to adjust to the surface profile of the substrate (e.g., [20,45,70]). This results in anisotropic material properties (i.e., the pads are soft during compression); however, those withstand high tensile stress [74,75]. The more spaced fibres of the arolium consequently would bend more efficiently under pressure and easily adapt to surface irregularities increasing adhesion [44]. The euplantulae feature relatively thinner and with more densely distributed fibres enhances protection against environmental conditions such as wear [76] and evaporation [44]. This enhanced resilience comes at the expense of reduced adaptability to surface irregularities.

As a frictional pad, the euplantula requires increased wear resistance, prioritizing it over optimal conformability to surfaces to withstand applied shear forces without undergoing degradation.

Similar morphological features have been previously described by Clemente and Federle [54] for the arolium and euplantulae of the cockroach *N. cinera*, by Bennemann et al. [71] for the arolium of the stick insect *C. morosus*, and by Schmitt and Betz [45] for the arolium and euplantulae of the cockroach *G. portentosa*.

The hollow spaces between fibres within the primary rod layer and the branching rod layer can also be important for adjusting the material properties of the attachment pads. Adhesive secretion kept within the spaces could impact the viscoelasticity of the pad, as well as its shape due to the internal pad pressure caused by the fluid. Spherical structures between the fibres, identified via SEM, could be indications for liquid residues (Figure 7C) (similar residues have been also identified by Gorb et al. in the euplantulae of *T. viridissima* [20]). In addition, the red CLSM autofluorescence signal within the euplantulae might have been caused by the adhesive fluid or by the exocrine cells (Figure 5C), assuming it contains organic molecules with a conjugated system of electrons caused by C=C double bonds [39,40]. The adhesive secretion within the primary rod layer and branching rod layer could work as a soft backing enhancing the conformability to the substrate and friction generation in contact with rough substrates [77].

Endocuticle layer 1
Another morphological difference between the arolium and euplantulae is observed in the endocuticle layer 1. In the arolium, the endocuticle layer 1 is thicker (arolium: ≈10 µm; euplantula: ≈5 µm) (Figure 3; Figure 5D) and more intensely stained with toluidine blue and Cason compared to that of the endocuticle layer 1 of the euplantulae (Figure 3; Figure 5). This difference potentially arises from the larger volume of the arolium, necessitating a stronger endocuticle layer 1 as a support for the primary and branching rod layers. Additionally, the parallel layer structure of the endocuticle layer 1 could give additional resistance against shear forces [78].

Exocrine cells
The exocrine cells of both attachment pads show multiple morphological similarities. Both exocrine cells display comparable staining patterns with toluidine blue and Cason. They possess a sizable nucleus containing numerous nucleoli, a substantial abundance of vesicles and hollow spaces, the absence of a discernible structural mechanism for product release (e.g., a duct), and the presence of a dedicated storage area for their respective products (e.g., the adhesive secretion reservoir).

Collectively, these distinctive features categorize the exocrine cells as exocrine cells type I [62]. Despite their morphological similarities, there are a few differences between the exocrine cells of the arolium and the euplantulae. The initial distinction is the presence of a wide basal layer in the arolium situated between the exocrine cells and the hemolymph (Figure 3; Figure 5). Although the euplantulae likely possess a very thin basal layer, similar to that found in *G. portentosa* [45], confirmation requires TEM analysis. The presence of the wide basal layer potentially augments the mechanical stability of the exocrine cells and ultimately of the arolium [79].

Another difference lies in the autofluorescence of both exocrine layers in CLSM. The exocrine cells of the arolium exhibit a stronger red autofluorescence signal, while those of the euplantulae display green autofluorescence (Figure 3 (arolium); Figure 5 (euplantulae)). Both attachment pads were separately scanned but under the same conditions and settings. Therefore, the difference in the autofluorescence signal could be the result of the two scans (i.e., surrounding material influencing the projected intensity) or be an indication of a difference in composition between the two cell aggregations.

Morphological investigations of the adhesive fluid of *M. extradentata* using cryo-SEM revealed different structures that the fluid can adopt, as well as slight differences between those of arolium and euplantulae [38]. It was postulated that these structures arise due to different mixing ratios of the fluid, and that the fluid can therefore fulfill different functions.

Our results remain ambiguous. The morphological similarities between the exocrine cells of both types of pads suggest that
both produce the same adhesive fluid, which is potentially differentiated by various mixing ratios or production rates. It is also possible that the difference in the autofluorescence indicates that the arolium and euplantulae produce different substances.

Schmitt and Betz [45] discovered no major morphological differences between the exocrine cells of the arolium and euplantulae of *G. portentosa* as well. This could be an indication that the adhesive fluid and its production may be similar between the two species.

Furthermore, the exocrine cell layer of the arolium is more strongly folded in comparison to that of the euplantulae (Figure 2 (aroilum); Figure 4 (euplantulae)). The enlarged surface could offer more exocrine cell area increasing the discharge area of the secretion, as well as allowing the pad to deform more easily, making it more resistant to mechanical stresses.

**Endocuticle layer 2**
The endocuticle layer 2 is strongly pronounced around the exocrine cells of the euplantulae, as evidenced by the darker staining with toluidine blue and Cason (Figure 5A, Figure 5B). In the arolium, however, the endocuticle layer 2 is not recognizable.

The wider endocuticle layer 2 in the euplantulae could be a structural feature that increases the resistance to shear forces as well as the stability of the attachment organ. A layer with similar properties, the inner cuticular band, has been previously observed in the arolium and euplantulae of *G. portentosa* by Schmitt and Betz [45].

**Adhesive secretion reservoir**
The endocuticle layer 1 and endocuticle layer 2 are separated by a confined space measuring \(10 \mu m\) in width, the adhesive secretion reservoir, which is slightly stained with toluidine blue and Cason in both the arolium and euplantulae (Figure 3; Figure 5). Based on the light staining with toluidine blue and Cason, the adhesive secretion reservoir probably consists of very loosely packed cuticle fibres which allow the adhesive secretion to be stored. Due to the potentially loose structure of the adhesive secretion reservoir, it is susceptible to rupture, whereby the actual size of the reservoir is difficult to determine. In addition to serving as a repository for the secretion, this reservoir could play an additional role in providing a pliant support structure when filled with adhesive secretion, thereby contributing to the stabilization of the respective attachment pad [77]. A morphologically similar adhesive secretion reservoir layer was also observed in the arolium and euplantulae of *G. portentosa*, as well as in the arolium of *T. viridissima* [45,80].

**Internal subdivision of the euplantulae**
The division of the euplantulae into four areas (Figure 4; Figure 7F) results in four independent volumes filled with hemolymph capable of generating internal hydraulic pressure. This pressure could potentially influence the shape of the euplantulae and therefore control the attachment process. Similar principles were discovered in the toe pads of tree frogs where the blood pressure maintains its shape [81], and in the arolium of ants where hemolymph pressure inflates them [82]. Dening et al. [83] showed in an artificial system that internal air-filled bladders can control attachment strength.

**Anti-adhesive structures**
The superficial layer of the connective pad is patterned in a hemispherical shape at the predominantly peripheral position towards the centre. This position suggests that such structures act as an adhesion- and friction-reducing system (anti-adhesive structures, Figure 7B) [86]. The hemispherical pattern reduces the contact area between the cuticle and the substrate, thus decreasing contact forces. Similar surface structures were observed in the wax coverage of plants where they decreased the attachment performance of insects [84]. A reduction of the contact area and the resulting reduced adhesion was shown by Wu et al. [85] for artificial structures. Reducing attachment could be helpful in the areas where such structures are found as they prevent the adhesion of folds in membranous cuticles in the regions of the connective pad. They might also reduce the risk of trapping contaminants in the inter-tarsomeric membranous region. The removal of particulate contaminants is very important as they are known to cause abrasive wear in the open insect joints [86]. Anti-adhesive surface structures in the periphery of the active working areas of the attachment pads could establish zones facilitating detachment. Such detachment movements are described for flies with hairy attachment pads [87], but would function similarly in smooth ones.

**Connective pad**
Our investigations have revealed a continuity of several underlying layers from beneath the euplantulae extending through the connective pad region. These layers encompass the hemolymph, exocrine cells, endocuticle layer 2, adhesive secretion reservoir, endocuticle layer 1, and superficial layer (Figure 6). Notably, the connective pad lacks both the primary rod layer and branching rod layer but exhibits an additional stratum of outer parallel cuticle. In CLSM, the exocrine cells exhibit green autofluorescence, whereas the procuticle emits blue autofluorescence, which is consistent with observations in the euplantulae.
Footprints of the connective pad revealed residues of the secretory fluid (pers. obs.). Given the identification as a soft cuticle, the structural attributes of the procuticle, and the presence of adhesive secretion, it is possible that the connective pad could participate in the attachment process.

Similar connective pad structures are present in insect species that also possess split euplantulae [88]. However, many euplantulae-bearing insect species do not have split euplantulae and therefore do not possess a connecting tissue [5,45,89-91], including several phasmid species [88,92].

**Transportation pathway of the pad secretion**

The schematic representation delineates the potential site of adhesive secretion production and its transportation from the hemolymph reservoir to the surface of the euplantula and arolium (Figure 8). The exocrine cells, situated in the epidermal tissue, obtain the educts for the adhesive secretion from the hemolymph. Both attachment pads exhibit exocrine cells with surface expansions into the hemolymph increasing the area for reactant uptake.

The adhesive fluid is secreted through pores in the endocuticle layer 2 [45] and accumulates in the adhesive secretion reservoir (indicated by the split arrow). Subsequently, the secretion traverses the endocuticle layer 1 via pores [45] and enters the primary rod layer. Within the primary rod layer and branching rod layer layers, the secretion fills the cavities between the rods (indicated by the split arrows), extending throughout the layers up to the superficial layer. The transportation of the adhesive
secretion to the surface is facilitated through pores in the superficial layer [64] (Figure 7C,E).

The cuticle layering and morphology of the arolium and euplan- tulae facilitate the absorption, storage, and distribution of the produced adhesive secretion within the attachment pads, enabling its transport to the surface. As mentioned above, the presence of the fluid secretion in these layers modulates the stability of the corresponding layers, potentially serving as a soft backing enhancing attachment on the substrate by maximizing the contact area [77].

Dirks and Federle [15] observed that the adhesive secretion volume in the phasmid C. morosus was completely depleted after approximately 7–10 consecutive press-downs (steps), with a subsequent restoration to its original volume taking approximately 15 min, indicative of a steady-state supply. The existence of multiple reservoirs (the adhesive secretion reservoir as well as the hollow spaces in both the primary rod layer and branching rod layer) suggests a continuous supply of adhesive secretion toward the surface, minimizing the likelihood of complete depletion of the attachment pad. Additionally, the denser cuticle rod structure of the branching rod layer may potentially restrict the flow of adhesive secretion, thereby reducing the risk of excessive fluid production.

Schmitt and Betz [45] also postulated a comparable transport pathway for adhesive secretions in the smooth attachment pads of G. portentosa. There, the adhesive secretion produced by exocrine cells type I is transported through a two-layered inner cuticle band via pores (comparable to the endocuticle layer 2) and accumulates in the secretion reservoir. It then passes through a layered cuticle via pores (comparable to the endocuticle layer 1) into a sponge-like cuticle where it fills the hollow cavities (comparable to the primary rod layer and branching rod layer). The final route to the surface is via pores in the ventral cuticle band and the epicuticle (comparable to the superficial layer in this study).

Conclusion
The examination of the ultrastructure and material composition of the tarsal attachment apparatus of the stick insect Medauroides extradentata yielded insights into the detailed structure of the two attachment pad types (arolium and euplan- tulae). Our findings revealed differences in the structure and material composition between them, indicative of their different roles during attachment. We proposed a potential pathway for the adhesive secretion from the exocrine cells to the surface and provided evidence suggesting the involvement of exocrine cells type I, which exhibit some variability between the arolium and euplan- tulae. For a more comprehensive understanding of the functional principles of both pad types, a detailed examination of their ultrastructure and testing of their material properties is required. Transmission electron microscopy and atomic force microscopy are ideal approaches for this purpose.

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Competing Interests
The authors declare no competing or financial interests.

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Julian Thomas: conceptualization; data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft. Stanislav N. Gorb: conceptualization; funding acquisition; methodology; project administration; resources; writing – review & editing. Thies H. Büscher: conceptualization; data curation; methodology; project administration; supervision; validation; writing – review & editing.

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All data that supports the findings of this study is available in the published article.

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**Functional fibrillar interfaces: Biological hair as inspiration across scales**

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**Abstract**

Hair, or hair-like fibrillar structures, are ubiquitous in biology, from fur on the bodies of mammals, over trichomes of plants, to the mastigonemes on the flagella of single-celled organisms. While these long and slender protuberances are passive, they are multifunctional and help to mediate interactions with the environment. They provide thermal insulation, sensory information, reversible adhesion, and surface modulation (e.g., superhydrophobicity). This review will present various functions that biological hairs have been discovered to carry out, with the hairs spanning across six orders of magnitude in size, from the millimeter-thick fur of mammals down to the nanometer-thick fibrillar ultrastructures on bateriophages. The hairs are categorized according to their functions, including protection (e.g., thermal regulation and defense), locomotion, feeding, and sensing. By understanding the versatile functions of biological hairs, bio-inspired solutions may be developed across length scales.

**Introduction**

Given the bottom-up approach that biology uses to create materials, fibrous structures formed by molecular chains are found everywhere. For example, internally in the form of collagen [1] and microtubules and microfilaments [2], and externally in the form of silk [3] and hair [4,5]. Among these prevalent, quasi-one-dimensional structures, here we loosely define biological “hairs” as high-aspect-ratio structures that are external and passive. This definition is loose yet intuitive. First, a structure must be on the exterior of an organism to be considered as “hair”. This excludes the internal one-dimensional structures...
such as microfilaments, veins, or bones. Second, in the definition presented here, “hairs” need to be passive, that is, the high-aspect-ratio structures must not be internally active. Obviously, this excludes organisms’ slender body parts, such as elephant trunks, the legs of mammals and insects, and the cilia and flagella of eukaryotic microorganisms. As a side note, flagella of eukaryotic cells (e.g., algae, protists, and sperms) and prokaryotic cells (bacteria) should not be confused. Eukaryotic flagella are essentially the same organelles as cilia, consisting of a well-organized microtubular backbone and orchestrated internal protein motors, whereas bacterial flagella are simply passive, stiff filaments. The passive nature of the hairs does not lessen their importance. They play a crucial role in mediating an organism’s interactions with the environment, serving various functions depending on their deformations, which are driven purely by their surroundings. Altogether, following the definition above, the structures covered in this review include the hair and fur of mammals, the feathers of birds, the trichomes of plants, the setae of arthropods, and the ultrastructures of single-celled organisms.

Figure 1A shows how the total hair mass $m_h$ scales with body mass $m_b$. For $m_h$, a material density of 1 g·cm$^{-3}$ was assumed. A relationship slightly exceeding isometry is observed, where $m_h \sim m_b^{1.16}$ with 95% confidence interval (CI) of (1.07, 1.15) for the exponent. For purely isometric scaling, if body mass decreases or increases by a factor of 100, then total hair mass decreases or increases by that same factor, respectively. Isometric scaling supports the fact that, with respect to certain characteristics, organisms are scaled copies of each other [6]. For example, as expected from isometry, the total surface area of a salamander was found to scale with $\sim m_b^{2/3}$ [7], and the same scaling was found for the total area of adhesive pads of animals within the same phylogenetic class, order, family, genus, and species [8]. However, hair mass deviates slightly from isometry, and it appears that larger organisms are more “hairy”. First, the exponent for power-law fits increases with size, as evidenced by comparing the fits for cells and phages, insects, mammals, and birds (see caption of Figure 1A). Second, from the inset of

![Figure 1: Scaling of hair across body size.](image)

(A) Scaling of hair mass $m_h$ versus body mass $m_b$. The dots represent data with different colors referring to mammals (blue) [5], birds (red) [9,10], insects (green) [5], and cells and phages (yellow) [11-23]. The dashed black line represents the best power-law fit for all of the data: $m_h \sim m_b^{1.16}$ with $R^2 = 0.98$ and 95% confidence interval (CI): (1.07, 1.15). The solid black line represents equal masses: $m_h = m_b$. The solid lines with different colors represent the best power-law fits for the different groups: mammals (blue) $m_h \sim m_b^{1.16}$ with $R^2 = 0.74$ and CI: (0.74, 1.61), birds (red) $m_h \sim m_b^{1.15}$ with $R^2 = 0.94$ and CI: (1.06, 1.34), insects (green) $m_h \sim m_b^{1.15}$ with $R^2 = 0.81$ and CI: (0.60, 1.70), and cells and phages (yellow) $m_h \sim m_b^{0.79}$ with $R^2 = 0.93$ and CI: (0.48, 0.92). The inset shows a plot of the ratio of hair mass to body mass $m_h/m_b$ as a function of body mass $m_b$. The ratio appears to increase with body mass following a Spearman rank test ($\rho = 0.55$, $p = 3.35 \times 10^{-5}$). The silhouettes mentioned below are from Noun Project. They are distributed under the terms of the Creative Commons CC BY 3.0 License, https://creativecommons.org/licenses/by/3.0, and attributed to the following creators: Andre Buand (phage), ProSymbols (mosquito), Creative Stal (butterfly), Laymilk (duck), and Pham Thanh Loc (beaver). (B) The eye of a sheep. Scale bar represents 10 mm. (C) The eye of a fruit fly and (i) close-up of its ommatidia. Scale bars represent 200 µm and (i) 20 µm, respectively. (D) Green microalgae and (i) close-up of its flagellum with mastigonemes. Scale bars represent 10 µm and (i) 1 µm, respectively.
Figure 1A, the ratio of hair mass to body mass $m_h/m_b$ is higher for larger organisms. A Spearman’s rank test supports this observation, with $\rho = 0.55$, which corresponds to an increasing trend between $m_h/m_b$ and $m_b$. Therefore, it seems that larger organisms dedicate more energy and resources to growing and maintaining hair. This finding motivates the following questions: (1) What are the purposes of hair? (2) How do these purposes vary with organism size?

For countless animal species, hairs are strategically placed throughout the body, varying in size and structure. Figure 1B–D show examples of various hairs found in mammals, insects, and micro-algae, respectively. Depending on their location and configuration, hairs serve a multitude of functions that can contribute to an organism’s homeostasis. The diversity of their function is exemplified by hair’s resistance to heat transfer in humans [4,24], and the role of hair in sensing mates by male mosquitoes [25]. Additionally, plants may exhibit hair-like fibrillar structures, such as the nanometer-thick mastigonemes on the flagella of microalgae [26] and the high-aspect-ratio, hair-like trichomes on plant surfaces [27]. Overall, to promote homeostasis in plants, animals, bacteria, and bacteriophages, fibrillar structures contribute to the following functions: protection (e.g., thermal insulation and defense), locomotion and feeding, and sensing. This review will present how biological hairs, or fibrillar structures, contribute to those functions across 20 orders of magnitude in organism mass and six orders of magnitude in hair thickness, from the nanometer-thick fibers on bacteriophages to the millimeter-thick hair and fur on mammals.

**Review**

**Protection**

Plants and animals often encounter potential danger in their surroundings. For example, extreme weather, such as precipitation and low temperatures, predators, and disease vectors. Because of their protruding, fibrillar structures serve as one of the first lines of defense against such dangers. They can protect against heat loss by providing insulation (Figure 2A), prevent the penetration of water through hierarchical superhydrophobicity (Figure 2B), or provide protection from predators or disease vectors through mechanical interactions (Figure 2C).

**Thermoregulation**

Regarding thermal regulation, mammals have evolved certain traits that differentiate them from other animals. In addition to regulating their temperatures by exploiting metabolic processes, mammals tend to be covered in dense coats of fur or hair. A key attribute of these fibrillar structures that promotes insulation is their air-filled center core [28].

Animal pelts and furs are still being utilized by humans as jackets and blankets in order to provide thermal insulation. The fur trade still rears 100 million animals annually, and millions of wild animals are caught in the U.S. every year for their fur [29]. While their continued use is ethically debatable, furs have presumably persisted because of their thermal insulation properties. In mammals, the thickness and packing density of hair arrays was found to coincide with the geometrical parameters that minimize convective heat loss, with the hair diameter $d$ scaling as $d \sim m_b^{1/2}$ [5,24].

In aquatic mammals, hair morphology, including shape and packing density, differ from terrestrial mammals in order to maintain a trapped air layer within the arrays of hair when submerged in water [30]. Hairs of aquatic animals have been found to be flatter, shorter, and packed in higher densities. Additionally, mammals that also rely on blubber for insulation, such as sea lions and walruses, were found to have lower hair-

Figure 2: Protection through hairs. Schematics showing (A) an array of hairs providing thermal insulation, (B) a superhydrophobic hairy surface with self-cleaning properties, and (C) hairs defending from unwanted interactions with other organisms. The silhouettes mentioned below are from Noun Project. They are distributed under the terms of the Creative Commons CC BY 3.0 License, https://creativecommons.org/licenses/by/3.0, and attributed to the following creators: madness stock (hot and cold thermometers), Petra Prgomet (bear), Manish (falling water drops), Parallel Digital Studio (pollen grains), and twist.glyph (bacteria).
packing densities \cite{30}. In extreme cases of reliance on blubber (e.g., in bowhead whales) instead of hair for insulation, arrays of hairs are limited to specialized regions where sensory information can be measured, for example, around the chin, lips, and blowhole \cite{31}.

While dense arrays of hair can trap a thermally insulating layer of air to protect the animal from cold temperatures, sparse arrays of hair can act like fins, which enhance heat exchange with the surroundings and help to cope with hot temperatures. When hair densities are low, such as in elephants, sparse hair arrays can help to shed heat \cite{32}. The hair density of elephants is around 0.03–0.07 hairs per square centimeter, which is more than three orders of magnitude sparser than the typical hair density on human head (200–300 hairs per square centimeter) \cite{32}.

Hairs on humans have also been reported to protect the skin from UVA and UVB radiation from the sun \cite{33}. UV radiation from the sun can not only heat up human skin but is also linked to skin cancers. Therefore, in mammals and birds, hairs provide protection from thermal effects, depending on their density, and from cancer-causing radiation. This demonstrates the multifunctionality of hairs, even within one species, such as humans.

When comparing fur and feathers, it has been found that feathers can outperform fur in protecting against solar radiation. In arid environments in Australia, the feathers of emus \textit{(Dromaius novaehollandiae)} prevent nearly all solar radiation from reaching the bird’s body, while the fur of red kangaroos \textit{(Macropus rufus)} prevents 75–85\% of the solar radiation from reaching the mammal’s body \cite{34}. It is thought that the deep coat of feathers protects from solar radiation, so the emus are able to reside in the open without needing to search for shade to cool down. In the ground hornbill \textit{(Bucorvus leadbeateri)}, lash-like feathers on the upper rim of their eyelids were found to provide shading from the sun to protect their corneas from intense sunlight \cite{35}.

At the scale of insects, setae may also contribute to thermoregulation. Bumblebees, which inhabit globally northern regions, possess dense arrays of setae on their thorax, while other species of bees inhabiting the tropics and hot deserts have very sparse arrays of setae \cite{36}. Such a stark difference is associated with the colder temperatures that bumblebees have to contend with. However, there are trade-offs in possessing dense arrays of fibrillar structures, that is, they contribute to increased aerodynamic drag. Wasps, which are predators that need to outpace their prey during flight, do not possess such insulating arrays of setae \cite{36}.

Finally, when an organism is extremely small, such as single-celled organisms and bacteriophages, thermoregulation is limited. Theoretically, the largest temperature difference that a cell with a diameter of 10 \mu m and calorimetric heat generation of 100 pW can experience is only \approx 10^{-5} \degree C \cite{37}. Additionally, even if a cell of the same size was capable of maintaining a 10-\mu m-thick air layer (with thermal conductivity of 3 \times 10^{-3} \text{ W·m}^{-1}·\text{K}^{-1}) along its surface, following steady-state one-dimensional heat conduction, it could still only experience a temperature difference of \approx 10^{-4} \degree C. Therefore, thermal insulation would have a negligible effect on thermoregulation at this scale. Instead, cells may be able to regulate their metabolic rates in response to changes in environmental temperatures \cite{38}.

**Wettability**

Superhydrophobic surfaces have the unique capability of preventing water from spreading; thus, they exhibit low wettability. In order to achieve superhydrophobicity, surfaces should have structural hierarchy and be composed of materials with low surface energy. The classic example of such a surface in nature is the lotus leaf \cite{39}, which possesses wax-covered microscopic pillars. The superhydrophobic surface is self-cleaning since water droplets bead up on the surface, and, when they roll off, they pick up any dirt or other particles and remove them from the leaf’s surface. This phenomenon was termed the “Lotus effect” and has been translated to the development of a self-cleaning paint called Lotusan\textsuperscript{\textregistered}.

Superhydrophobic, fibrillar surfaces are also present in animals, such as insects, spiders, and geckos. Similar to plants, these structures help to maintain a clean body surface by enabling the rolling-off of water, which collects unwanted contaminants, or by providing low adhesion. Such structures are typically found on body parts where contamination is common, such as adhesive pads \cite{40}, or where cleanliness is crucial for survival, such as insect wings \cite{41}.

Hairs provide more ways to prevent or clean contamination. For a dedicated review on the topic, please see \cite{5}. However, we will mention some of the cleaning functions of hairs here. Hairs around the eyes of mammals (eyelashes) and on the eyes of insects (interommatidial setae) have been found to minimize the deposition of particle-laden contaminants through aerodynamic interactions \cite{42,43}. Hairs on honey bees have been found to facilitate both the collection and removal of pollen grains through the geometries of the hair arrays on their eyes and grooming appendages \cite{44}. Mammalian fur effectively sheds contaminants because the hair deflects when exposed to a fluid flow. This deflection generates a shear flow that removes contaminants \cite{45}.
In addition to superhydrophobicity, in certain water plants, such as Salvinia spp., specialized structures have been observed to combine superhydrophobicity and superhydrophilicity [46]. In these plants, the fiber-like structures have hydrophilic tips, while the rest of the structure is hydrophobic. The combination of these different wetting properties enables the plants to maintain a stable layer of air while underwater. The hydrophilic tips pin the water surface so that it does not penetrate the fiber array and, thus, trap an air layer directly on the plant’s surface.

While the combination of hydrophilic tips with superhydrophobic structures enables stable air film retention underwater, some animals exploit superhydrophobic surfaces for various functions on or under the water surface. For example, water striders (Gerridae spp.) possess superhydrophobic structures on their limbs, which help them locomote on the water surface [47]. Similarly, groups of ants form rafts to float on water and escape flooded regions [48]. This function relies on the wetting properties of their cuticle and its substructures. When underwater, spiders, such as the diving bell spider (Argyroneta aquatica), and insects, such as aquatic bugs and beetles, use hydrophobic hairs to trap air and form an air bubble that encompasses their body [49,50]. Insects, such as the green dock beetle (Gastrophysa viridula), trap air between the adhesive fibers on their footpads when walking underwater to generate adhesion [51].

Mechanical protection
While hairs provide protection via their thermal and chemical properties, they also offer protection based on their mechanical properties. Hairs are typically made of stiff materials, such as keratin and chitin, that have Young’s moduli of the order of gigapascals, comparable to typical values for wood. Therefore, they can be quite robust to mechanical stimuli from external sources.

Mammals possess guard hairs, that are interspersed with the rest of their body hairs or furs. These hairs are distinctly thicker than the rest and have been reported to help provide protection to the rest of the mammal’s coat from abrasion [52]. Guard hairs are also involved in mechano- and thermosensation [53,54]. In addition to guard hairs, some mammals have developed spines or quills to provide protection from predators [52]. These are typically thicker but still made of the protein complex keratin.

Plants also make use of fibrillar structures to provide defense against predators. These structures are known as trichomes and vary in morphology and density. While trichomes may also secrete chemicals to warn predators, they can impale insects and their larvae when they have a hooked morphology or even sting herbivores [55]. It has been observed that plants with higher densities of trichomes suffer less from insect herbivory. Also, there is a reduced incidence of internal egg laying by insects with ovipositors [55].

Locomotion and feeding
While hairs can help to protect organisms and to promote homeostasis, strategically placed arrays of hairs are crucial for locomotion through various mediums, such as granular soil, air, and water. By possessing hairs on appendages, organisms across wide length scales are capable of enhancing their locomotory performance. Examples are reversible adhesion in geckos (Figure 3A), anchoring in burrowing earthworms (Figure 3B), flying in bristled-wing insects, such as thrips, (Figure 3C), and swimming in unicellular microorganisms, such as microalgae (Figure 3D).

Climbing
Adhesive hairs have been observed on the foot and toe pads of insects, spiders, and geckos [56]. These hairs range in diameter from hundreds of nanometers in geckos, to micrometers in...
spiders, to tens of micrometers in insects. The hairs are capable of generating adhesive forces through either capillary interactions, when an adhesive fluid is present [57], or intermolecular interactions, when no adhesive fluid is present [58]. In addition to adhesive forces that enable inverted climbing, the hairs can also generate friction forces whenever the animals climb on vertical surfaces [59].

For geckos, these adhesive hairs are referred to as setae, which branch into spatulae at the tips [60]. The branching ensures a high density of hairs to generate high adhesive forces [61]. With advances in fabrication techniques at the micrometer scale, gecko-inspired adhesive hairs have been developed, which are capable of generating adhesion without the use of glues or fluids [62,63].

For insects, the adhesive hairs are around one order of magnitude thicker than those of geckos, and they rely on fluid secreted by the hairs to generate adhesive forces. The hairs on insect footpads can vary in morphology, and these variations have been linked to their functions [57,59]. Green dock beetles (G. viridula) have been observed to possess three distinct types of adhesive hairs: discoidal, spatula-shaped, and sharp-tipped. The males possess adhesive hairs with discoidal tips, which are capable of generating large adhesive forces [57]. They are hypothesized to be used by males to attach securely to females when mating [59]. Both males and females possess adhesive hairs with spatula-shaped and sharp tips. The spatula-shaped tips enable reversibility of adhesion, while the sharp-tipped hairs are used for generating friction [59].

For microorganisms, while gravity is less of a concern, adhesive hairs are no less useful than for insects and larger animals. Microalgae, such as Chlamydomonas reinhardtii (10 µm in size), possess >100 nm long thin hairs on their flagella [11], which help them to attach to surfaces to glide or to attach with other cells to mate. Bacteria, such as Pseudomonas aeruginosa (2 µm in size), use thin filaments (up to several micrometers long), known as pilus (type IV), to attach to surfaces and, in effect, "tow" themselves around on the surface [64]. Bacteriophages (~100 nm in size) rely on their tail filaments to attach to their hosts [21,22].

Burrowing

The use of hairs to generate frictional forces is not unique to animals that climb. Hair-like setae on the skin of earthworms aid in burrowing by increasing friction and providing anisotropic anchoring [65-67]. When burrowing, the earthworm mainly uses cavity expansion to create a burrow. It expands some segments of its body to anchor itself, while elongating other segments to push through the soil [68].

This kind of motion is called peristaltic motion since the coordination of expansion and elongation of segments resembles a wave traveling through the worm’s body. It is similar to the motions exhibited by intestines during digestion [69]. The body expansion (increased cross-sectional size) and elongation are controlled by the worm’s circular muscles. When the worm stiffens its circular muscles, the corresponding body segments expand and the setae are erected, helping the worm to anchor tighter to the surrounding soil. Meanwhile, when the circular muscles relax, the setae deflect and interact less with the soil. This anisotropic burrowing has been realized in a bio-inspired burrowing soft robot [70].

Flying

Flying organisms span about eight orders of magnitude in mass, ranging from the smallest known parasitoid wasp (Dicopomorpha echmepterygis, 2.5 × 10⁻⁸ kg) to the great bustard (Otis tarda, 21 kg). The fluid regimes experienced by these organisms vary greatly with scale, from a highly viscous, laminar environment at the smallest sizes to an inertial, turbulent environment at the largest sizes. Thus, the locomotory appendages of these organisms vary widely with size and the fluid regime they experience.

Structurally and developmentally, feathers are analogous to hair. Bird feathers, like hair, are complex structures made primarily of keratin. Despite approximately 200 million years of independent evolution, feathers and hair follicles share numerous structural similarities, including the presence of a dermal papilla and a dermal sheath [71]. However, unlike hair, feathers also have a dermal pulp, which is essential in growth and regeneration during feather cycling [72]. Much like the mammalian hair cycle [4], feathers are conveniently repaired during grooming and replaced seasonally during molt.

Feathers are highly structured and exhibit self-similarity. They are comprised of a central rachis, which gives rise to barbs. These barbs then branch into barbules, which, in turn, branch into smaller hook-like projections called barbicels. These barbicels cause the barbs to interlock, resulting in a continuous feather surface with relatively low air transmissivity [73]. Many birds have feathers that exhibit lobate cilia and hooked rami, which hook and loop together to prevent gaps between feathers [74].

Beyond forming the aerodynamic surfaces necessary for flight, feathers often exhibit species-specific adaptations. For example, owls have serrations on their leading-edge primary feathers, which are known to reduce noise during flight by mitigating flow instabilities [75,76]. Conversely, many birds use their feathers to produce sound through a variety of mecha-
nisms, including aeroelastic flutter and mechanical rubbing [77,78].

Around one third of birds, notably crepuscular and nocturnal species, such as nighthawks, have facial bristles that resemble mammalian whiskers [79]. These bristles are hypothesized to act as tactile sensors and may aid in prey handling, collision avoidance, foraging, or navigation, as well as provide eye protection [80,81].

Bats are the only mammals capable of powered flight. Their wing membrane is covered with short hairs, which act as tactile airflow sensors [82,83]. The hairs grow sparsely on the membrane of the wing and in fringes on the wing’s leading edge. The neurons associated with these hairs can discriminate airflow directionality, and exhibit the highest firing rate when airflow is reversed, which is associated with slow flight and stall [84]. Indeed, when these hairs are removed, bats alter their flight performance by increasing speed and reducing their turning radius [85].

The membranous wings of insects are covered with bristle (or “hair”) sensilla that act as airflow sensors [86,87]. In Odonata wings, bristle sensilla account for approximately 60% of all wing sensors [88]. In some cases, these bristle sensilla are highly tuned for specific airflow conditions. For example, tests on the silkworm moth (Bombyx mori) revealed that their bristle sensilla responded to vibrating air currents but not to constant flow [89]. It is hypothesized that the height of these bristles matches the height of the boundary layer, but further aerodynamic testing is necessary [88].

The smallest insects, such as beetles, thrips, and parasitoid wasps, possess wings made entirely of bristles [90-96]. The bristles (or setae) of these wings support flapping flight by reducing inertia, enhancing aerodynamic performance, and facilitating their deployment (i.e., folding and unfolding). The wing acts as a leaky paddle, and can produce 66–96% of the aerodynamic drag force of an equivalent membranous wing [94]. Conventionally, the competition between inertial and viscous forces is captured by the Reynolds number (Re), and a large Re indicates a dominant role of inertia. At Re = 4–60, the effects of viscosity are significant, and inertial forces are relatively weak [97,98]. Consequently, traditional steady-state lift-based flight, as observed in larger organisms, is not possible. Thus, miniature insects use unsteady aerodynamics through a combination of wing flapping, wing clap-and-ting, and recapture of vortices to generate lift and thrust through the manipulation of air resistance (drag) [99]. In essence, the very smallest insects move by rowing through the air, generating drag much like a paddle. The bristles are also known to improve control of the boundary layer and delay stall via the generation of leading-edge vortices.

Swimming

For microorganisms, whose body sizes typically range from \(10^{-7}\) to \(10^{-4}\) m and who live predominantly in water, “inertia is totally irrelevant” [100]. While Re is around \(10^5\) for a flying eagle or a swimming whale, it is \(10^{-3}\) to \(10^{-5}\) for moving microbes. A thought experiment gives a straightforward illustration to such drastic distinction [101]. Imagine that an animal flying or swimming at high speed suddenly freezes the motion of its body parts (wings, fins, or flukes), how long would it continue to travel through the medium? While displacement for an eagle or a whale can continue for some time and distance, typically, an Escherichia coli bacterium (3 µm long, swimming at \(10\) µm·s\(^{-1}\)) will stop immediately, that is, within \(10^{-6}\) s and \(10^{-10}\) m [100].

In this viscosity-dominated regime, because there is no inertia to depend on, microorganisms must constantly deform body parts in a non-time-reversible fashion to swim. Therefore, swimming efficiency depends on the order (or pattern) in which deformations take place. Three types of patterns are the most common: (1) rotation of a corkscrew-like tail found in archaea [102,103] and bacteria [15,104], (2) travelling waves along filaments (flagella) adopted by sperm cells and some algae [15], and (3) cyclic beating pattern consisting of a power stroke of large amplitude and a recovery stroke of small amplitude (similar to the arm movement during breaststroke swimming), which is adopted by microalgae [105] and ciliates [15].

In these locomotory patterns, microbial hairs are consistently involved in drag force generation. The flagella of archaea and bacteria are themselves passive hairs and are driven by protein motors at the base. Hair-like ultrastructures, or mastigonemes, on eukaryotic flagella/cilia comprise helical glycoproteins (=10–20 nm thick) and lack a membrane [106]. They can be either stiff or flexible. Flagella with thick and stiff hairs (tubular mastigonemes) are sometimes referred to as the “tinsel” type [12,107]. These stiff hairs help to increase the effective surface area of flagella and, thus, enhance swimming speed [15]. Moreover, the stiff hairs help to reverse the resultant swimming direction when travelling waves patterns are employed [108]. For example, the smooth flagellum of sperm [109] and the tinsel-like flagellum of golden algae Ochromonas [12] beat in the same pattern, featuring waves travelling away from the cell body. In Ochromonas, this results in a swimming direction towards the waves’ travelling direction, while sperm cells swim towards the opposite direction. This modulation effect has already inspired designs of swimming microrobots [110].
The role of thin and flexible hairs (fibrous mastigonemes) is still, to some extent, enigmatic. These soft hairs may appear in a range of number densities, from ca. 1 per micrometer of length of flagellum in *Phytophthora* [107] and *Ochrononas* [12], over ca. 10 per micrometer in the green algae *C. reinhardtii* [26], to 10^2–10^3 per micrometer in *Euglena* [111]. At least for the hair density found in *C. reinhardtii*, they do not help the cell to swim faster [26]. Nevertheless, without these hairs, swimming in *C. reinhardtii* is interrupted by frequent and sudden turns [26]. A possible explanation for this is that the fibrous hairs are involved in sensory functions, which may be crucial for stable, controlled swimming [112].

Microbial hairs commonly serve multiple roles at the same time. Hence, one should avoid understanding these hairs’ existence from a single, locomotory perspective. While the flagella of *E. coli* is most obviously an apparatus for swimming motility, it can also help the cell to attach to a surface and act as a sensor thereafter [113]. Intriguingly, even after attachment, having motile flagella still matters for the cell as it appears to enable sensing of substrate stiffness [114]. In addition to flagella, other hairs of *E. coli* include the type-I pili (frimbriae) and type-IV pili [113]. Collaboration between these hairs also helps the cell. When approaching a solid surface, the cells become trapped as they move in circular orbits because of hydrodynamic effects [101, 104]. While staying close to the surface may be beneficial as it facilitates surface attachment and, hence, the formation of bacterial biofilms, remaining in circular trajectories hinders the cell’s ability to explore the surface thoroughly. Thus, possibly with the help of the other hairs, *E. coli* near the surface can transiently attach to the surface to break the circular trajectories, thus, pushing their exploration efficiency close to the theoretical optimum [115].

Developing tools with one-dimensional structures is arguably the most basic and economical (materials-wise) solution for microorganisms. In this light, the “hairs” are their available tools, where most tools happen to look alike. This is the primary reason why microbial hairs defy easy classifications. Future research linking form and function in microbial hairs may lead to a better understanding of their evolution, as well as providing inspiration for the development of functional fibrillar structures at the micrometer and nanometer scales.

**Filter feeding**

Locomotion is key for searching for food, and hairs may also serve crucial roles in feeding, particularly via filtering. Filter feeding uses a porous material to capture prey and nutrients in fluid flows. Dense arrays of hairs may serve as the porous material that captures the food, separating it from the surrounding flow or from other unwanted objects. The capturing can occur via sieving, where food larger than the gaps between the fibers gets trapped, or through hydrodynamic interactions that transport food to the fiber surface, where it can stick and become trapped [116].

At the largest scales, baleen whales (Mysticeti) use keratinous fibers, or baleen, in their mouths instead of teeth to filter and capture prey [117–119]. When feeding, the whales use three different strategies, depending on their species. Bowhead and right whales (Balaenidae) use ram filter feeding where they continuously swim through groups of prey with their baleen exposed, collecting prey while the filtered water exits through an opening in the posterior of their mouths. Rorqual whales (Balaenopteridae) use lunge feeding where they swallow mouthfuls of prey and water and then push the seawater out through their bristled baleen in order to isolate the prey for swallowing. Grey whales (*Eschrichtius robustus*) use suction filter feeding [119].

At the smaller scales, aquatic insects of the orders Ephemeroptera, Trichopteram, and Diptera use filter feeding to consume organic matter from their aqueous environment [120]. The fibrillar filters used by insects include setae, mouth brushes, and fans. The setae are present around the mouthparts or forelimbs and may be lined with arrays of smaller fibers, called microtrichia. The mouth brushes are dense arrays of fibers present on the lower jaw, or labrum. Fans are arrays of fibers that can be opened (splayed) and closed. The captured organic matter in the fans is consumed by sweeping the mandibles over the closed fans [120].

Choanoflagellates are unicellular organisms that use filter feeding. They drive fluid flow through a conical filter consisting of microvilli with diameters of 100–200 nm, spaced 200–700 nm apart [121, 122]. While the microvilli contain actin and myosin, which together enable motility during escapes and help to transport trapped organic matter for consumption [123], they function passively when filtering organic matter. The structure driving the fluid flow through the filter remains elusive. A flagella alone does not seem to provide enough flow to explain the experimentally observed filtering rates. However, it has been proposed that a flagellar vane, which behaves like an undulating wall, could induce enough flow through the conical filter [122].

**Sensing**

Perceiving the environment using sensory organs in order to respond to stimuli is vital for survival in animals [124]. Hairy receptors are a type of sensing organ that exists widely across nature. They are systematically distributed throughout the surface of the bodies of organisms and play an important role in
reacting to external stimuli in order to perceive the environment, such as external touch (Figure 4A), odor (Figure 4B), temperature (Figure 4C), and humidity [125-127]. Hairy receptors can be classified into several types according to their various functions and sensing modes, such as mechanoreceptors, chemoreceptors, thermoreceptors, and hygroreceptors. While there are different types of hairy receptors, depending on their location and type of stimulus they sense, they all generate electrical signals through their sensory cells and transmit the signals to the nervous system in order to paint a picture of the outside world or determine body or appendage orientation [128].

**Mechanosensation**

Hairs, as mechanical receptors, are capable of perceiving and distinguishing a multitude of external stimuli such as touch, vibration, or fluid flows [129,130]. The mechanosensation of hairs relies on the sensory cells at their base. When the hair is deflected by mechanical forces, the membrane potential of the sensory cells is altered, and an electrical signal is sent to the nervous system. By receiving, analyzing, and finally reacting to the signal, the organism is able to respond to changes in the surroundings [131].

Mechanical perception via hair is important for living organisms across length scales and evolutionary backgrounds. Cats, for instance, can sense the position, shape, and texture of objects by moving their whiskers, and can even use whiskers to sense the direction and speed of air flow to help them move in the dark or catch prey [132]. A review of hairy sensation in mammals can be found here [133]. Spider appendages [134], cockroach antennae [135], and cricket cerci [136] possess hairs capable of detecting delicate vibrations, airflow, and interactions with various objects, enabling them to locate their prey, evade obstacles, or detect potential dangers [130,137]. Airflow sensors with bio-inspired, fibrillar structures based on the working principles of cricket cerci, which, when clustered in arrays, aid in detection of oscillating flows following “viscous coupling” [138], have been developed [139].

At the microscopic scale, microalgae, such as *C. reinhardtii*, may utilize the hair-like ultrastructures, or mastigonemes, on their flagella to sense fluid flow while swimming [26]. The mastigonemes have been observed to be anchored to a channel protein that shows ion conductivity, and the mastigome-channel protein complex may provide mechanical gating to sense deflections of the mastigonemes caused by fluid flow [112]. Additionally, for bacteria, *E. coli*, their passive flagella have been linked to sensing the material stiffness of surfaces they attach to [114].

Clusters of hairs, or hair plates, on the limbs of insects are used for proprioception or to sense the orientation and motion of the limbs, which helps in their control of locomotion [131]. Furthermore, many insects, such as bees, can enhance their foraging speed by utilizing hairy mechanical receptors to detect physical characteristics such as food viscosity and texture [140,141]. Mechano-sensing with hairs, regardless of stimulus, relies on deflections of the hairs triggering deformations to the sensory cells they are attached to.

**Chemoreception**

In addition to touch, hairs are able to sense their less-immediate environment by detecting the presence and alteration of chemicals [142], which differs from the way hairs sense touch and vibration. The binding of receptor proteins on sensory cells to chemicals in the air or solution initiates a sequence of biochemical reactions, resulting in the production of electrical signals,
which are then transmitted to the nervous system. The brain interprets these electrical signals as a specific scent or flavor after they are processed by the nervous system [143].

Arthropods, including spiders [144], ants [145], and bees [146], possess chemical receptors on their limbs and antennae that detect chemicals in their surroundings, enabling them to locate sustenance, recognize their species, and avoid danger [147]. Moth antennae possess dense arrays of hairs, which have been found to interact with surrounding airflow in order to enhance diffusion of chemicals to the antennae for detection [148]. Based on this knowledge, bio-inspired, fibrillar chemical sensors have been developed [149,150]. Furthermore, insects can utilize hairs to sense atmospheric carbon dioxide [151,152]. The human scalp follicles also possess an olfactory perception and can even stimulate hair growth upon exposure to a specific fragrance [153].

Thermosensation

Hair can also act as a temperature sensor, helping organisms to choose the right temperature environment to keep their body thermally stable. The receptors typically have a short hair that protrudes through a small hole to interface with the environment, also known as a peg-in-pit sensillum [154]. The protruding hair-like receptors help to absorb thermal radiation, since the penetration depth of infrared radiation into insect cuticle is quite shallow [155]. Additionally, the hair-like sensillum possesses electron-dense filaments that may improve absorption [154]. Leaf-cutting ants (Atta vollenweideri), for instance, can utilize the temperature receptors of their appendages to detect intense heat outside their nests as indicators of where to locate food [156]. Cave-dwelling crickets (Tachycines plumiopedella) rely on thermosensation to detect temperature gradients and locate appropriate habitats in the environment, utilizing hair receptors on their labial palps [157]. In plants such as the Venus flytrap (Dionaea muscipula), heat was observed to trigger closure of their trap [158]. At the base of their trigger hairs, there are sensory cells that may be triggered by either mechanical or thermal energy [158].

Hygrosensation

Studies have also shown that hairs exhibit heightened sensitivity to changes in humidity levels, enabling arthropods to discern variations in air humidity with remarkable precision. There are three potential mechanisms for hygrosensation with hairs or sensilla: (1) Changes in humidity may cause changes in the volume of the sensilla, which could mechanically trigger sensory cells. (2) For hollow sensilla, the external humidity could cause lymph fluid to evaporate, and the change in fluid volume may trigger sensory cells. (3) Changes in humidity could cause changes in temperature of the sensilla and trigger thermoresponsive sensory cells [159]. These sensilla are distributed across the body, including antennae, legs, and other appendages. Insects such as locusts [160] and beetles [161] utilize hygroscopeceptors on their antennae to detect humidity fluctuations in their environment. Similarly, arachnids such as the harvestman (Heteromithobates discolor) also possess hygroscopeptive sensilla on their legs [162].

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