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/nanoscopic 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/nanoroughness 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/nanorough 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.

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 brake 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 Chrysolina 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, I. 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 fastuosa male beetle

General morphology

The tarsus of C. fastuosa 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, Gastrophysa 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

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**Figure 2**: SEM micrographs of attachment organs of a Chrysolina fastuosa male beetle. (a) Tarsus with pretarsus, dorso-lateral view. (b) The first (basal) proximal tarsomere (T1), ventral view. CL, claw; S1, setae with discoidal tips; S2, setae with pointed tips; T1-T3, three proximal tarsomeres. Arrows point to the distal direction. Scale bars: 200 μm (a) and 50 μm (b).
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
insects pooled together (i.e., in experiments with all waxy plant surfaces) and for data obtained from five insect individuals on each plant surface (species) separately. Original results on the forces and time in the case of pooled data are presented in Figure 6, whereas for the second case (separate plant species), graphs in Figure 7 show the force and time values normalized to the corresponding ones obtained in the first experiment on glass gl1.

Considering force data obtained from all insect individuals and all waxy plant surfaces tested (pooled data), we found a highly significant reduction (ca. 24-fold in average) of the maximum traction force $F_{\text{max}}$ on the waxy plant surfaces compared to those obtained in the corresponding first (control) force measurements on the glass substrate gl1 (paired $t$-test: $t = 26.286$, $p < 0.001$) (Figure 6a). The maximum traction forces $F_{\text{max}}$ from the second experiment on glass gl2 (performed immediately after tests on a waxy plant surface) were significantly lower than those from the first experiment on glass gl1 in all beetles (paired $t$-test: $t = 5.451$, $p < 0.001$) (Figure 6a). Also the comparison of the first peaks of the traction force $F_{\text{peak1}}$ measured from the force–time curves obtained in the first and second experiment on glass (gl1 vs gl2) showed significantly lower values in the second experiment gl2 (paired $t$-test: $t = 5.962$, $p = 0.033$) (Figure 6b). To reach the maximum traction force values, all insects needed significantly more time during the second experiment on glass gl2 compared with the first experiment on glass gl1 (paired $t$-test: $t = 2.203$, $p = 0.033$) (Figure 6c).

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 *A. negundo* 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 gl2. 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; $F_{\text{peak}1}$, value of the first peak of the traction force; $T_{F_{\text{max}}}$, time needed to reach the $F_{\text{max}}$ value.

Figure 6: Maximum traction force $F_{\text{max}}$ (a), first peak of the traction force $F_{\text{peak}1}$ (b), and time $T_{F_{\text{max}}}$ 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 $F_{\text{peak}1}$, and significantly longer times $T_{F_{\text{max}}}$ 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 $F_{\text{peak}1}$). 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

$F_{\text{max}}$, Fmax, maximal traction force; $F_{\text{peak}1}$, value of the first peak of the traction force; $T_{F_{\text{max}}}$, time needed to reach the $F_{\text{max}}$ value.
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>
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*p, probability value; $t$, test statistics; $^*$, significant difference.

ability of C. fastusosa 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 $F_{\text{max}}$. 

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\textit{L. serriola} regarding the first peak of the traction force $F_{\text{peak}}$, and \textit{L. germanica} regarding the time needed to reach the maximum traction force $T_{F_{\text{max}}}$. 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 \textit{A. vulgaris} and \textit{C. majus}) caused inconsiderable pad contamination and, in turn, did not significantly affect further beetle attachment.

\section*{Conclusion}

Traction experiments with tethered male individuals of the \textit{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 \textit{A. negundo} and \textit{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.

\section*{Experimental Plants}

Nine plants species from different plant families were used in the experiments: \textit{A. negundo}, \textit{Aloe vera} (L.) Webb. & Berth. (Asphodelaceae), \textit{Aquilegia vulgaris} L. (Ranunculaceae), \textit{Brassica oleracea} L. (Brassicaceae), \textit{Chelidonium majus} L. (Papaveraceae), \textit{Chenopodium album} L. (Chenopodiaceae), \textit{Iris germanica} L. (Iridaceae), \textit{Lactuca serriola} Torner (Asteraceae), and \textit{Trifolium montanum} L. (Fabaceae). Young stems (\textit{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.

\section*{Insect}

The leaf beetle \textit{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.

\section*{Scanning electron microscopy}

To visualize the waxy plant surfaces and attachment devices in the \textit{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].

\section*{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 $F_{\text{peak}}$, and the time $T_{F_{\text{max}}}$ 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 (g11), (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 \( T_{f_{\text{max}}} \) needed to reach the maximum traction force for the comparisons between g11 and plant and between g11 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).

**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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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 transport between different types of smooth attachment pads located on the same tarsus is scarce, especially in Phasmatodea. 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 adhesive 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 Phasmda.

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 tarsomeres. The proximal four tarsomeres bear one euplantula each, whereas the fifth tarsomere additionally carries the pretarsus including the arolium (see Figure 1B, Figure 1C). The five tarsomeres 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 OsO4 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 glycine. 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.
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

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**Figure 2:** Sections of the arolium visualized with different imaging techniques. The internal ultrastructure of the arolium 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. (B) Cross section stained with toluidine blue, light microscopy. (C) SEM. (D) CLSM. For images (C) and (D) the arolium had to be dissected. All images are similarly positioned: the ventral side of the arolium is located at the bottom of the picture. Ap = attachment pad; as = adhesive secretion reservoir; cu = cuticle; ep = epidermal cells; ex = exocrine cells; ha = hair/seta; he = hemolymph.
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 radiosity 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.

ible cuticle (fc). 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 revealed 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].

**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 *Tettigonia 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 fulfil. 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. (C) 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 μm 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 arolim 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 arolia 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

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**Figure 8:** Scheme of the arolium (left) and euplantula (right) of *M. extradentata*. Schematic representation of the transportation of the adhesive secretion from its point of origin towards the substrate. A description of the production and transportation pathway of the adhesive secretion is provided in the text. as = adhesive secretion reservoir; bl = basal layer; br = branching rod layer; e1 = endocuticle layer 1; e2 = endocuticle layer 2; ex = exocrine cells; he = hemolymph; pr = primary rod layer; sf = superficial layer. Blue arrows indicate the pathway of the adhesive secretion in the respective pad type. Brightening of the arrows indicates a reduction in the amount of adhesive fluid.
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 Medau- roidea eastradentata 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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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.

References


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 bacteriophages. 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 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-
packing densities [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 [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 [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) [32].

Hairs on humans have also been reported to protect the skin from UVA and UVB radiation from the sun [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 (Dromaius novaehollandiae) prevent nearly all solar radiation from reaching the bird’s body, while the fur of red kangaroos (Macropus rufus) prevents 75−85% of the solar radiation from reaching the mammal’s body [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 (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 [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 [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 [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 µm and calorimetric heat generation of 100 pW can experience is only ≈10⁻⁵ °C [37]. Additionally, even if a cell of the same size was capable of maintaining a 10-µm-thick air layer (with thermal conductivity of 3 × 10⁻³ W·m⁻¹·K⁻¹) along its surface, following steady-state one-dimensional heat conduction, it could still only experience a temperature difference of ≈10⁻⁴ °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 [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 [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®.

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 [40], or where cleanliness is crucial for survival, such as insect wings [41].

Hairs provide more ways to prevent or clean contamination. For a dedicated review on the topic, please see [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 [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 [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 [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 mechanoe- 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 bars. These bars then branch into barbules, which, in turn, branch into smaller hook-like projections called barbicels. These barbicels cause the bars 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 facilitating their deployment (i.e., folding and unfolding). The wing acts as a leaky paddle, and can produce 66–96% of the drag 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].

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 Ochromonas[12], over ca. 10 per micrometer in the green algae C. reinhardtii[26], to 10^2–10^3 per micrometer in Eudorina[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 preying upon 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, Trichoptera, 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 hygrometers. 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 mastigone–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]. Mechanosensing 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 plumipedella) 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 hygroreceptors on their antennae to detect humidity fluctuations in their environment. Similarly, arachnids such as the harvestman (Heteromitobates discolor) also possess hygroreceptive sensilla on their legs [162].

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Author Contributions

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References


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Investigation on drag reduction on rotating blade surfaces with microtextures

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Abstract
To enhance the aerodynamic performance of aero engine blades, simulations and experiments regarding microtextures to reduce the flow loss on the blade surfaces were carried out. First, based on the axisymmetric characteristics of the impeller, a new simulation method was proposed to determine the aerodynamic parameters of the blade model through the comparison of flow field characteristics and simulation results. Second, the placement position and geometrical parameters (height, width, and spacing) of microtextures with lower energy loss were determined by our simulation of microtextures on the blade surface, and the drag reduction mechanism was analyzed. Triangular ribs with a height of 0.2 mm, a width of 0.3 mm, and a spacing of 0.2 mm exhibited the best drag reduction, reducing the energy loss coefficient and drag by 1.45% and 1.31% for a single blade, respectively. Finally, the blades with the optimal microtexture parameters were tested in the wind tunnel. The experimental results showed that the microtexture decreased energy loss by 3.7% for a single blade under 57° angle of attack and 136.24 m/s, which was favorable regarding the drag reduction performance of the impeller with 45 blades.

Introduction
In order to survive, organisms in nature have undergone billions of years of evolution; their body structures have been adapted to the current environment and exhibit special functions on biological surfaces [1]. For the purpose of drag reduction, valuable inspiration can be derived from rapidly moving animals, such as the “denticles” found on the surface of shark skin, which enable high-speed swimming [2], as well as the texture of bird feathers [3]. The phenomenon of drag reduction can also be observed on the surface of plants. For example, there is a superhydrophobic structure on the surface of lotus leaves [4]. A thin gas film captured by the superhydrophobic structure creates a slip interface between gas and liquid, which effectively improves the
drag reduction and antifouling performance of lotus leaves [5]. However, the structures on biological surfaces are rather complex and not directly applicable in practice. Therefore, researchers have explored the drag reduction mechanisms through replication or imitation of the microtexture found on biological surfaces; it was found that the contribution of microtextures to drag reduction primarily occurs within the boundary layer [6,7]. Lang et al. [8] constructed rectangular and sinusoidal grooves with 2 mm in width, 3 mm in depth, and 1 mm in spacing, thus mimicking the transverse grooves on the surface of dolphin skin. They observed the effect of the grooves on flow separation and boundary layer using digital particle image velocimetry. Xiao et al. [9] analyzed the drag reduction mechanism of bionic microtextures and constructed simplified V-shaped, trapezoidal, and wavy ribs by grinding. Experimental and simulation studies on aeroengine blades with such microtextures showed that the drag reduction performance of wavy ribs is better than that of the other two structures. Tian et al. [10] pointed out that, because of the complexity of microstructures on the shark skin surface, it is difficult to use a uniform method to characterize the skin surface. Triangular grooves or rectangular grooves can be used to simplify the microstructures on the shark skin surface to study the effects on hydrodynamics and aerodynamics.

Within these extremely small structures, a low-speed, stable fluid flow exists, which can mitigate turbulences and enhance the stability of fluid motion within the boundary layer, resulting in a reduction of frictional drag [11]. Based on the above principles and for large-scale manufacturing, researchers imitated and simplified the microtextures of biological surfaces to form the structures with different sectional shapes, such as triangles, trapezoids, and ellipses [12,13], as shown in Figure 1.

The drag reduction effect of biomimetic microtextures can reduce friction and turbulence pulsation on blade surfaces, thus, improving the aerodynamic performance of blades [14]. The research on drag reduction of microtextures on blade surfaces can be traced back to the 1980s. In 1982, Walsh et al. [15] from NASA Langley Laboratory conducted a pioneering microtexture study on surfaces. Their experiments focused on longitudinal grooves with various shapes, revealing that symmetrical V-shaped grooves exhibited a remarkable drag reduction effect at low flow rates. The highest drag reduction rate (DRR) attained was 8%. Chamorro [16] studied fans with a grooved surface and found that, under certain operating conditions, the drag reduction effect of local coverage on the textured blade surface surpassed that of a complete covering. Additionally, they designed microgrooves of various sizes on the suction surface to achieve the optimal drag reduction effect. Zhang et al. [17] proposed a method to determine the placement position of microtextures by using finite element analysis. The suggested microtextures were arranged on blade surfaces and exhibited drag reduction compared to smooth blades. Mischo et al. [18] improved the cooling capability of turbine blades by adding grooves to the blade tips of axial turbines. Experimental and numerical simulation results showed that the addition of grooves increased turbine efficiency by 0.2% and 0.38%, respectively. In order to reduce aerodynamic losses in turbines caused by tip leakage, Parkash et al. [19] added grooves at the blade tips and verified their effectiveness through computational fluid dynamics (CFD) simulations. After the incorporation of grooves, the turbine efficiency improved by 0.1% to 0.2%.

It is evident that arranging microstructures on blade surfaces can optimize the aerodynamic performance of the blades, thereby achieving energy savings. However, the rational arrangement of microstructures on blade surfaces also requires investigation, as the shape, size, and placement position of microstructures can all affect drag reduction performance [20,21]. Wu et al. [22] investigated the effect of different sizes of triangular grooves on the drag of NACA 0012 airfoils, finding an optimal DRR of 9.65% when the microstructure dimensions were $s = h = d = 0.1$ mm. Liang et al. [23] arranged various sizes of triangular microstructures on rotating disks and found through comparative analysis that microtextures with
modeling of microtextures on blade surfaces. As well as the equipment and processes in the actual processing of microtextures. Finally, the wind tunnel experiment platform used to measure the drag reduction of the microtextured blades is described.

**Impeller**

The research object of this paper is the impeller of an axial flow compressor, which consists of a hub and blades [26]. In order to generate high pressure, axial flow compressors typically comprise multiple stages of impellers, as shown in Figure 2a. When the compressor is working, the air flow is driven by the rotation of the impeller from the inlet to the outlet, as shown in Figure 2b. The working conditions of the impeller mainly include the rotational speed and the environmental conditions (temperature and pressure) at inlet and outlet. The impeller considered in this paper has 45 blades and is designed to operate at a rotational speed of 2880 rpm. The operating environment for the impeller is at standard temperature (25 °C) and pressure (101325 Pa).

**Methods**

This section introduces the modeling of microtextures on blade surfaces, as well as the equipment and processes in the actual processing of microtextures. Finally, the wind tunnel experiment platform used to measure the drag reduction of the microtextured blades is described.

**Modeling of microtextures on blade surface**

To determine the geometry and position of the microtextures on the blade surface, a new simulation method is proposed based on the axisymmetric characteristics of rotating machinery. The complete flow domain model of the compressor was simplified into a single flow channel model, so that the flow field on the smooth blade surface could be obtained quickly and accurately. A flow diagram consisting of four steps is shown in Figure 3.
Step 1
The compressor model has rotational symmetry, and each blade is uniformly installed on the compressor. Therefore, the compressor model was evenly divided according to the number of blades to obtain a calculation domain model including a single blade and a single flow channel. In the simulation setup, the walls on both sides of the channels were set as periodic boundaries, which can simulate the flow domain with symmetry and make up for the calculation error caused by the simplified model. Through the above simplification, the calculation cost can be greatly reduced while ensuring calculation accuracy.

Step 2
The microtexture placement position is determined according to the flow field of the smooth blade. Flow separation occurs during high-speed air flow over the curved blade surface. Here, a reasonable arrangement of microtexture can effectively improve the drag reduction. Therefore, CFD was employed to simulate a single channel model of the blade and the flow separation region was obtained. In order to verify the accuracy of the simulation calculation, the theoretical calculated value of the angle of attack was compared with the simulation results.

Step 3
First, the drag reduction performance of four microtextures was compared by numerical simulations to determine the geometric type with the optimal drag reduction. Then, different widths ($w$), spacings ($s$), and heights ($h$) of the microtextures were compared to determine the scale range with drag reduction. In the simulation setup, the initial conditions and the flow domain are consistent with the single flow domain of the blade. The coefficient of friction and the DRR from the simulation results were compared to determine the geometric types and size ranges of the microtexture with drag reduction performance.

Step 4
According to the flow field on the smooth blade surface, groove and rib microtextures were arranged before and after the flow separation point on the suction surface. The difference between grooves and ribs will be described in the passage referring to step 4 in the Results and Discussion section. Also, based on the microtexture types and size range determined in step 3, the drag reduction results of grooves and ribs with different size parameters were compared to determine the combination of microtexture parameters with the best drag reduction performance. This
parameter combination was employed for machining microtextures on the blade surface, and the microtextured blade was placed in the wind tunnel for experiments.

Details of step 1 and step 3
Here, the details about the simulation setup method and the determination method of microtextures in step 1 and step 3 of the proposed method are described. To enhance readability, the relevant results from steps 2, 3, and 4 will be analyzed in the Results and Discussion section.

Step 1
The whole impeller with 45 blades was divided evenly, that is, each single flow domain occupies an 8° fan-shaped flow channel, as shown in Figure 4. The length of the flow channel is 300 mm, and the bottom radius and top radii are 300 and 410 mm, respectively.

In the simulation setup, the \( k-\varepsilon \) model with enhanced wall treatment, which has better prediction results for rotation, boundary layer separation with large back pressure gradient, and backflow phenomena, was used for turbulence modeling in Ansys® CFX, Release 2020 R2 [28]. The boundary conditions were set according to the working conditions of the impeller, that is, the inlet of the impeller was set as the velocity inlet with a value of 75 m/s; the outlet was set as the pressure outlet with the value of 101325 Pa. Periodic boundary conditions were applied on both sides of the flow domain, and the upper and lower walls were no-slip walls, as shown in Figure 4. According to the above impeller parameters, we used the method of speed triangle [29] to calculate the theoretical value of the 0.5 blade height (span = 0.5). The relative airflow velocity was determined to be 130.67 m/s, with an angle of attack of 54.97°.

The height of the first layer mesh should be calculated according to the requirement of \( y^+ \) (dimensionless distance from the wall), which is determined according to each turbulence model requirements. In order to obtain good mesh quality and meet the \( k-\varepsilon \) model requirements used in this paper, \( y^+ \) needs to be between 1 and 5 [30]. In this paper, the height of the first layer mesh is 0.005 mm, and \( y^+ = 1.5 \), as shown in Figure 5.

Step 3
The method of dimensionless size was used to determine the size range of microtextures with drag reduction performance [31]. In this section, the microtexture sizes (i.e., \( h \), \( w \), and \( s \)) were determined according to the boundary layer theory as shown in Figure 6.
The dimensionless size calculation formula of microtextures with drag reduction performance are as follows [32]:

\[
s^+ = \frac{su_x}{\nu}, \quad (1)
\]

\[
h^+ = \frac{hu_x}{\nu}, \quad (2)
\]

\[
w^+ = \frac{w_u}{\nu}, \quad (3)
\]

\[
u_u = \left(\frac{\tau_w}{\rho}\right)^{1/2}, \quad (4)
\]

\[
m = \rho v, \quad (5)
\]

where \(\mu\) is the dynamic viscosity, \(v\) is the kinematic viscosity, \(u\) is the average flow velocity, \(u_x\) is the wall stress shear rate, \(\tau_w\) is the wall shear stress, and \(\rho\) is the density.

The flow condition around the flat plate wall can be determined by the dimensionless local Reynolds number.

\[
\text{Re}_x = \frac{\rho u_x}{\mu}, \quad (7)
\]

where \(x\) is the distance from the inlet along the fluid flow direction. For \(\text{Re}_x < 3 \times 10^5\), the flow in the boundary layer is laminar, and the following equation yields \(\delta_L\):

\[
\delta_L = 4.96 \times \left(\frac{v}{\text{ux}}\right) = 4.96\text{Re}_x^{-1/5}. \quad (8)
\]

The flow is turbulent if \(\text{Re}_x > 3 \times 10^6\), and the thickness of \(\delta_T\) is calculated as:

\[
\delta_T = 0.37 \times \left(\frac{v}{\text{ux}}\right) = 0.37\text{Re}_x^{-1/5}. \quad (9)
\]

The flow in the boundary layer is transitional when \(\text{Re}_x\) is between \(3 \times 10^5\) and \(3 \times 10^6\). The turbulent area is selected for the arrangement of microtextures. Therefore, Equation 9 is entered into Equation 6:

\[
\tau_w = 0.0225\rho u^2 \left(\frac{v}{u\delta}\right)^{1/4}, \quad (6)
\]

\[
\tau_w = 0.029\rho u^2 \left(\text{Re}_x\right)^{-1/5}. \quad (10)
\]

Entering Equation 10 into Equation 4 gives:

\[
u_u = 0.17u\text{Re}_x^{-1/10}. \quad (11)
\]

Entering Equation 11 into Equation 1, Equation 2, and Equation 3, respectively, yields:
Based on Equations 12–14, the dimensionless sizes corresponding to microtextures under different $Re_x$ can be obtained; $Re_x$ needs to be determined according to the flow velocity and the characteristic dimensions ($x$) of the calculation domain. For our blades, $u$ is 75 m/s, and the maximum value of $x$ is 300 mm, as shown in Figure 4. Therefore, according to Equation 13 and the range of $h^+$ given in Table 1, the values of $h$ of the microtextures with drag reduction performance were first determined. The range determination for $w$ and $s$ in Table 1 will be described in the Results and Discussion section.

Experimental method
The overall process of experiments involves machining the microtextures on the blade surface and conducting experiments with the microtextured blades in a wind tunnel. The Results and Discussion section will give details about the determined microtexture types and sizes.

Experimental equipment
A list of equipment used in the experiment is shown in Table 2.

Microtexture processing of blade surfaces
A JDGR400-A13S five-axis CNC machine tool was used to process the blade and the microtextures; the processing steps are shown in Figure 7. First, the blank blade was installed in the machine tool, the size of the blank is $h \times l \times w = 140 \times 100 \times 25 \text{ mm}^3$. Second, the end milling tool was used to mill the blank roughly to improve the processing efficiency. Third, the high-quality blade models were obtained by finishing the rough blade models. Finally, the microtextures were machined on the blade surfaces; this step was also divided into roughening and finishing because the end milling tool would break if the tool with a smaller radius was used to process the microtextures directly. The tool parameters used in different processing stages are given in Table 3.

Wind tunnel platform
The experiment was conducted at the intermittent wind tunnel platform at the College of Energy and Power Engineering, Nanjing University of Aeronautics and Astronautics. Pictures and a schematic diagram of the wind tunnel test platform are shown in Figure 8. Figure 8b shows that the wake measurement device consisting of a three-hole probe, a motor, and a guide rail. Ten probes were utilized to measure the wake, while the

### Table 1: Microtexture parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>direction</td>
<td>spanwise, longitudinal</td>
</tr>
<tr>
<td>type</td>
<td>grooves, ribs</td>
</tr>
<tr>
<td>height ($h$)</td>
<td>$5 &lt; h^+ &lt; 25$ [15]</td>
</tr>
<tr>
<td>width ($w$)</td>
<td>$&lt; 3h$</td>
</tr>
<tr>
<td>spacing ($s$)</td>
<td>$&lt; 3h$</td>
</tr>
<tr>
<td>position</td>
<td>front, back</td>
</tr>
</tbody>
</table>

### Table 2: Overview of the equipment used in the experiment.

<table>
<thead>
<tr>
<th>Equipment name</th>
<th>Model</th>
<th>Purpose</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>five-axis CNC machine tool</td>
<td>JDGR400-A13S</td>
<td>processing of blades and microtextures</td>
<td>Beijing Jingdiao Technology Group Co., LTD, China.</td>
</tr>
<tr>
<td>flat end mill tool</td>
<td>ø 8 x 37 x ø 8 x 81 x 3F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>processing of blades</td>
<td>Shanghai Mituo CNC Equipment Co., Ltd, China.</td>
</tr>
<tr>
<td>ball end mill</td>
<td>ø 0.3 x 0.6 x ø 4 x 50 x 2F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>roughening of microtextures</td>
<td>MISUMI (China) Precision Machinery Trading Co., Ltd</td>
</tr>
<tr>
<td>ball end mill</td>
<td>ø 0.2 x 0.3 x ø 4 x 50 x 2F&lt;sup&gt;a&lt;/sup&gt;</td>
<td>finishing of microtextures</td>
<td>MISUMI (China) Precision Machinery Trading Co., Ltd</td>
</tr>
<tr>
<td>trinocular stereo microscope</td>
<td>JSZ6S</td>
<td>observing the processed blades</td>
<td>Nanjing Jinsong Optical Instrument Co., Ltd, China.</td>
</tr>
<tr>
<td>three-dimensional video microscope</td>
<td>KH-7700</td>
<td>high-precision 3D imaging</td>
<td>QUESTAR Corporation, Japan</td>
</tr>
<tr>
<td>intermittent wind tunnel</td>
<td>customized equipment</td>
<td>aerodynamic performance testing</td>
<td>Nanjing Power Tiger Electromechanical Technology Co., Ltd, China.</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ø (tool diameter in mm) × (cutting edge length in mm) × ø (shank diameter in mm) × (overall length in mm) × (number of flutes)/F.
Figure 7: The manufacturing procedure of the microtextured blade.

Table 3: Tool parameters in different machining stages.

<table>
<thead>
<tr>
<th>Process stage</th>
<th>Tools</th>
<th>Tool radius (mm)</th>
<th>Number of flutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>roughening of blades</td>
<td>flat end mill</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>finishing of blades</td>
<td>flat end mill</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>roughening of microtextures</td>
<td>ball end mill</td>
<td>0.15</td>
<td>2</td>
</tr>
<tr>
<td>finishing for microtextures</td>
<td>ball end mill</td>
<td>0.1</td>
<td>2</td>
</tr>
</tbody>
</table>

Figure 8: Experimental platform and schematic diagram. (a) Wind tunnel test platform. (b) Three-hole probe measuring device. (c) Schematic diagram of the wind tunnel test platform.

motor facilitates control and adjustment of their position. The total pressure (\(TP\)), static pressure (\(P\)) and velocity of the air flow (\(V\)) in the experiment were obtained by the three-hole probe measuring device. These results can be calculated according to Equations S5–S8 in Supporting Information File 1 to obtain Mach number (\(Ma\)) and energy loss coefficient (\(\xi\)). The
angle of attack can be set by controlling the motor and, thus, turning the disc (Figure 8c). In Figure 8c, the inlet of the test platform is connected with the high-pressure gas source, which is a 100 m³ high-pressure gas tank with a maximum of 25 atm; the air extraction source is a 200 m³ vacuum tank with a minimum of 0.1 atm.

Experimental steps
To ensure the accuracy of experiments, the velocity and angle of attack for blade heights of 0.25, 0.5, and 0.75 were selected to carry out multiple tests to verify the drag reduction effect of the microtextured blade. The specific steps of the experiment are as follows: (1) preparation of two blades in contrast, that is, one smooth blade and another blade with a microtexture on the surface; (2) test the smooth blade first; adjust the wind tunnel flow velocity and the angle of attack to 123.98 m/s and 52.8°, respectively; (3) measurement of the TP and P at the inlet and outlet, respectively, and calculation of ξ and observation of the wake loss distribution; (4) change of velocity and angle of attack to 130.67 m/s and 54.8°, respectively, and continuation according to step 3; (5) change of velocity and angle of attack to 137.54 m/s and 57.0°, respectively, and continuation according to step 3; (6) installation of the blades with microtextures and repetition of steps 3–5. The results obtained from the above steps will be discussed in the “Results of the experiments” section.

Results and Discussion
Determined microtextures
The details about the results obtained in steps 2–4 of the simulation method described in the Methods section are given here. In step 2, the simulation results of a single impeller blade were analyzed to determine the flow field characteristics around the blade, such as angle of attack, velocity, and air flow state. As shown in Figure 9a, we sliced the calculation domain to analyze the simulation results and selected the green plane at 0.25 of the total length of the flow domain in streamwise direction. In the radial direction, three curved surfaces with spans of 0.25, 0.50, and 0.75 of the blade height, progressing from the bottom to the top, were chosen for analyzing the velocity distribution at the intersection between these curved surfaces and the green plane. Figure 9b indicates an increase in the peripheral speed of the blade as the radius increases. The average velocity at span = 0.5 is 131.5 m/s, with an error of only 0.6% from the theoretical value of 130.67 m/s. This result serves as evidence supporting the reliability of the simplified simulation method; hence, the velocity in the local area simulation was set at 130.67 m/s.

In Figure 10, two-dimensional flow streamlines of the curved surface at span = 0.25, 0.5, and 0.75 are analyzed. The relative velocity angle of airflow and blade changes as the blades rotate. A comparison of simulation results is shown in Table 4, the error between the simulation value and the theoretical value of the angle of attack is only 0.31%. Hence, the flow field of the smooth blade surface at span = 0.5 is further analyzed.

Table 5 presents the resistance results of the smooth blade, where the total drag (Td) in the direction of airflow was divided into pressure drag (Pd) and friction drag (Fd). The primary impact of the microtextures is to modify the flow state of the boundary layer near the wall, reducing of Fd. For our blade, the contribution of Fd is small, accounting for only 2.39% of Td. Thus, this paper primarily focuses on assessing the influence of microtextures on system energy loss.

From the leading edge to the trailing edge of the blade, the pressure surface exhibits a favorable pressure gradient, whereas the suction surface presents an adverse pressure gradient. This observation is complemented by Figure 11a, which illustrates that the turbulent kinetic energy (k) on the blade surface is small. The position of X = 0 mm in Figure 11a corresponds to the

![Figure 9: Single blade and simulation results. (a) Slices of the calculation domain. (b) Simulation results of flow velocity at span = 0.25, 0.5, and 0.75. Figure 9a used courtesy of ANSYS, Inc.](image-url)
Figure 10: Simulation values of attack angle at (a) span = 0.25, (b) span = 0.50, and (c) span = 0.75. x is the axial direction, y is the radial direction, and z is the rotation direction of the blade. Images used courtesy of ANSYS, Inc.

Table 4: The comparison of simulated and theoretical values of the angle of attack.

<table>
<thead>
<tr>
<th>Surface</th>
<th>Simulation values (%)</th>
<th>Theoretical values (%)</th>
<th>Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>span = 0.25</td>
<td>52.30</td>
<td>52.80</td>
<td>0.95</td>
</tr>
<tr>
<td>span = 0.50</td>
<td>54.80</td>
<td>54.97</td>
<td>0.31</td>
</tr>
<tr>
<td>span = 0.75</td>
<td>56.50</td>
<td>57.00</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Table 5: Aerodynamic parameters of the smooth blade.

<table>
<thead>
<tr>
<th>Type</th>
<th>$P_d$ (N)</th>
<th>$F_d$ (N)</th>
<th>$T_d$ (N)</th>
<th>$F_d/T_d$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>smooth</td>
<td>1.3288</td>
<td>0.0325</td>
<td>1.3613</td>
<td>2.39</td>
</tr>
</tbody>
</table>

highest point of the blade surface (point B in step 4 of Figure 3). Observing the distribution of $k$ on the periodic boundary shows that $k$ is zero in the front region of the blade ($X \leq 0$ mm). However, $k$ begins to rise sharply from $X = 40$ mm, indicating that boundary layer separation at this position generates turbulence. The peak of $k$ indicates that this position corresponds to the center of the turbulent vortex. According to Figure 11b, the turbulent vortices manifest on the adverse pressure surface within the system. Therefore, the microtextures were arranged on the adverse pressure surface.

Based on Figure 11, the air flow separation is initiated at $X = 30$ mm. As a result, the suction surface can be divided into two regions (front and back) at $X = 30$ mm, which serves as the critical point to discuss the drag reduction performance of the microtexture.

In step 3, the geometric types and size ranges of the microtextures were determined. Because of the high flow velocity on the blade surface, transverse microtextures would significantly increase the projected area in the flow direction, leading to a drastic increase in $P_d$. Therefore, longitudinal microtextures were considered here. Because the flow projection area in longitudinal microtextures is small, $F_d$ contributes the most to $T_d$. $F_d$ is related to the friction drag coefficient and the surface area, while the shape of the microtextures affects the surface area and surface flow. Therefore, microtextures of four shapes was investigated in this paper, as shown in step 3 of Figure 3.
The drag reduction of the above four microtextures was simulated using the same simulation settings as in step 1. The flow velocity and the angle of attack were obtained from the results at span = 0.5, which were 130.67 m/s and 54.8°, respectively. The DRR of the four microtextures is shown in Figure 12. First, by comparing the rectangle and triangle 1 with the same values of \( h, w, \) and \( s \), it is evident that the DRR of the triangle is greater than that of the rectangle. This is because the surface area of the rectangle is larger than that of triangle 1, resulting in higher frictional drag of the rectangle. Comparing triangle 2, trapezoid, and oval microtextures, it can be seen that the DRR of triangle 2 is higher than that of the other two using the same size parameters.

Figure 13 shows the coefficient of friction \( (C_f) \) of the microtexture surface. The \( C_f \) at the bottom of the microtextures is smaller because of the low speed of the fluid, which also confirms that the \( C_f \) is affected by Re. From Figure 13, it can be observed that there is a significant variation in \( C_f \) at the corners.

Figure 12: DRR results of four microtextures with different sizes. The formula for calculating DRR is shown in Equation S4 of Supporting Information File 1.

Figure 13: Comparison of coefficient of friction \( (C_f) \) on the surface of (a) rectangle and triangle 1, (b) triangle 1 and triangle 2, (c) triangle 2 and trapezoid, and (d) triangle 2 and oval. The source of \( C_f \) is shown by the red line in the upper left corner of each panel.
of the microtextures. Inside the grooves, $C_f$ is smaller because the airflow velocity is lower. According to Figure 13c, there is little difference in the $C_f$ distribution between the triangular and trapezoidal surfaces. The average values of $C_f$ on microstructured surfaces are shown in Table 6; triangle 2 has the lowest average $C_f$. Therefore, after comprehensive analysis of Figure 12, Figure 13, and Table 6, the triangular microstructure was chosen to be machined in the blade surface.

The range of $h$ has been discussed in the Methods section. In order to determine the ranges of $w$ and $s$, different values of $w$ and $s$ of the triangular microtexture were chosen, and the DRRs were compared, as shown in Table 7 and Table 8.

Table 7 shows the change of DRR for different $w$ when $h$ and $s$ of the triangular microtexture are fixed values. It can be clearly seen that the microtexture increases the resistance (DRR < 0) when $w/h = 3$, and the microtexture has the best drag reduction performance when $h = 0.2$ mm, $w = 0.3$ mm, and $s = 0.3$ mm. Therefore, one of the requirements for the triangular microtexture with drag reduction is to meet the condition of $w/h < 3$. Table 8 shows the influence of different $s$ on the DRR. The maximum value of DRR is $-5.5138\%$ when $s = 0$ of the triangular microtexture, and the DRR gradually decreases with the increase of $s$. The triangular microtexture exhibits drag reduction under the condition of $s/h < 3$. In summary, the microtexture under the flow conditions described in this paper exhibits drag reduction only when $w/h < 3$ and $s/h < 3$.

In step 4, construction and comparison of different microtextures were carried out. To explore the effect of the microtextures on flow field and resistance, the grooves and ribs were arranged at the front and back sections of the suction surface, respectively, as shown in step 4 of Figure 3. According to the

<p>| Table 6: Average values of $C_f$. |</p>
<table>
<thead>
<tr>
<th>Microtexture</th>
<th>Rectangle</th>
<th>Triangle 1</th>
<th>Triangle 2</th>
<th>Trapezoid</th>
<th>Oval</th>
</tr>
</thead>
<tbody>
<tr>
<td>mean value of $C_f$</td>
<td>4.323</td>
<td>4.2000</td>
<td>3.8230</td>
<td>3.9906</td>
<td>5.6677</td>
</tr>
</tbody>
</table>

<p>| Table 7: The influence of the w of the triangular microtexture on the resistance. |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>$h$ (mm)</th>
<th>$w$ (mm)</th>
<th>$w/h$</th>
<th>$s$ (mm)</th>
<th>$F_s$ (10$^{-5}$ N)$^a$</th>
<th>$F_m$ (10$^{-5}$ N)$^b$</th>
<th>DRR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.5</td>
<td>0.3</td>
<td>5.957</td>
<td>5.930</td>
<td>-0.4459</td>
</tr>
<tr>
<td>W2</td>
<td>0.2</td>
<td>0.15</td>
<td>0.75</td>
<td>0.3</td>
<td>6.689</td>
<td>6.633</td>
<td>-0.8533</td>
</tr>
<tr>
<td>W3</td>
<td>0.2</td>
<td>0.2</td>
<td>1.0</td>
<td>0.3</td>
<td>7.415</td>
<td>7.317</td>
<td>-1.3114</td>
</tr>
<tr>
<td>W4</td>
<td>0.2</td>
<td>0.3</td>
<td>1.5</td>
<td>0.3</td>
<td>8.853</td>
<td>8.673</td>
<td>-2.0360</td>
</tr>
<tr>
<td>W5</td>
<td>0.2</td>
<td>0.4</td>
<td>2.0</td>
<td>0.3</td>
<td>10.300</td>
<td>10.181</td>
<td>-1.1593</td>
</tr>
<tr>
<td>W6</td>
<td>0.2</td>
<td>0.6</td>
<td>3.0</td>
<td>0.3</td>
<td>13.090</td>
<td>13.192</td>
<td>0.0147</td>
</tr>
</tbody>
</table>

$^a$ $F_s$ is the total resistance of the smooth wall from the simulation results; $^b$ $F_m$ is the total resistance of the microtextured wall from the simulation results.

<p>| Table 8: The influence of the s of the triangular microtexture on the resistance. |</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>$h$ (mm)</th>
<th>$w$ (mm)</th>
<th>$s$ (mm)</th>
<th>$s/h$</th>
<th>$F_s$ (10$^{-5}$ N)$^a$</th>
<th>$F_m$ (10$^{-5}$ N)$^b$</th>
<th>DRR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>0.2</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
<td>4.361</td>
<td>4.121</td>
<td>-5.5138</td>
</tr>
<tr>
<td>S2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.10</td>
<td>0.50</td>
<td>5.866</td>
<td>5.683</td>
<td>-3.1191</td>
</tr>
<tr>
<td>S3</td>
<td>0.2</td>
<td>0.3</td>
<td>0.15</td>
<td>0.75</td>
<td>6.613</td>
<td>6.427</td>
<td>-2.8093</td>
</tr>
<tr>
<td>S4</td>
<td>0.2</td>
<td>0.3</td>
<td>0.20</td>
<td>1.00</td>
<td>7.359</td>
<td>7.172</td>
<td>-2.5400</td>
</tr>
<tr>
<td>S5</td>
<td>0.2</td>
<td>0.3</td>
<td>0.30</td>
<td>1.50</td>
<td>8.853</td>
<td>8.673</td>
<td>-2.0360</td>
</tr>
<tr>
<td>S6</td>
<td>0.2</td>
<td>0.3</td>
<td>0.40</td>
<td>2.00</td>
<td>10.346</td>
<td>10.167</td>
<td>-1.7255</td>
</tr>
<tr>
<td>S7</td>
<td>0.2</td>
<td>0.3</td>
<td>0.60</td>
<td>3.00</td>
<td>13.333</td>
<td>13.155</td>
<td>-1.3335</td>
</tr>
</tbody>
</table>

$^a$ $F_s$ is the total resistance of the smooth wall from the simulation results; $^b$ $F_m$ is the total resistance of the microtextured wall from the simulation results.
flow field information from the smooth blade, the velocity in the front section of the suction surface is faster. The placement of ribs here increases the projection area, resulting in the increase of $P_d$, which will lead to advanced transition and separation of the flow; hence, the groove structure needs to be arranged in the front section. In contrast, the back section of the suction surface already exhibits separated boundary layers and turbulent vortices, and the ribs closer to the vortex have a more significant impact on the flow of the vortex. The ribs were arranged in the back section of the blade suction surface to optimize the lifting effect on the vortex. The results of drag reduction performance of microtextured surfaces are shown in Table 9.

Table 9 shows that adding microtexture changes the force on the blade. Compared with the back section of the suction surface, the drag increase and loss coefficient changes caused by the microtextures in the front section are more pronounced. Case 1 to case 3 indicate an increase in system energy loss without drag reduction effect. Moreover, a linear relationship exists between the rate of drag change and the height of the microtexture as the height directly influences the projected area and surface area. Ribs located in the back section of the blade exhibit a drag reduction effect, which is independent of the rib height.

The surface pressure distribution at the front end of the textured blade is shown in Figure 14a. It is evident that grooves substantially influence the pressure distribution, with more significant impact observed as the height of the microtexture increases. Conversely, the pressure distribution trend of the groove surface with a height of 0.1 mm resembles that of a smooth surface. Therefore, the groove has little effect on drag and $\xi$.

According to Figure 14b, the grooves arranged in the front section of the blade suction surface lead to an increase in turbulent kinetic energy. This results in an earlier increase in turbulence intensity, indicating the premature separation of the boundary layer and an associated increase in energy loss. From both the perspective of resistance changes and energy loss, grooves do not effectively contribute to drag reduction performance.

In Table 9, the microtextures were arranged at the back of the blade suction surface, which explicitly affects the DRR and $\xi$.

### Table 9: Simulation results of different types of microtexture in different regions on the blade suction surface.

<table>
<thead>
<tr>
<th>Group</th>
<th>Region</th>
<th>Type</th>
<th>$h$ (mm)</th>
<th>$w$ (mm)</th>
<th>$s$ (mm)</th>
<th>DRR (%)</th>
<th>$\eta_\xi$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>case 1</td>
<td>front</td>
<td>grooves</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.23</td>
<td>1.70</td>
</tr>
<tr>
<td>case 2</td>
<td>front</td>
<td>grooves</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>6.52</td>
<td>26.88</td>
</tr>
<tr>
<td>case 3</td>
<td>front</td>
<td>grooves</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>18.36</td>
<td>42.13</td>
</tr>
<tr>
<td>case 4</td>
<td>back</td>
<td>ribs</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>$-1.31$</td>
<td>$-1.37$</td>
</tr>
<tr>
<td>case 5</td>
<td>back</td>
<td>ribs</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>$-1.17$</td>
<td>$-1.43$</td>
</tr>
<tr>
<td>case 6</td>
<td>back</td>
<td>ribs</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>$-1.16$</td>
<td>$-1.09$</td>
</tr>
</tbody>
</table>

$^a$The change rate of energy loss coefficient from Equation S5 in Supporting Information File 1.

Figure 14: (a) Static pressure and (b) turbulent kinetic energy distribution of blade surfaces with different groove parameters.
In order to explore the drag reduction characteristics of ribs on the back, the rib parameters were further analyzed. The specific simulation results are shown in Table 10.

As shown in Figure 15a, the resistance increases with the height of the ribs. However, this relationship is not linear and is influenced by the coupling effect of the microtexture on $P_d$ and $F_d$. The highest DRR of $-1.31\%$ is obtained when $h = 0.1$ mm, while the lowest energy loss coefficient is observed when $h = 0.2$ mm. As shown in Figure 15b, the DRR is the highest when $w = 1.5h$, basically the same as for $w = 0.5h$, and the energy loss coefficient is the highest when $w = 1.5h$. We therefore select cases 8–10 ($w = 1.5h$) to research the spacing parameter further. According to Figure 15c, the spacing exhibits the same effect on the DRR and energy loss coefficient. Moreover, the drag reduction effect is optimal when $s = h$.

### Table 10: Simulation results of ribs with different parameters arranged at the back section of the blade surface.

<table>
<thead>
<tr>
<th>Group</th>
<th>Region</th>
<th>Type</th>
<th>$h$ (mm)</th>
<th>$w$ (mm)</th>
<th>$s$ (mm)</th>
<th>DRR (%)</th>
<th>$\eta_l$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>case 4</td>
<td>back</td>
<td>ribs</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>-1.31</td>
<td>-1.37</td>
</tr>
<tr>
<td>case 5</td>
<td>back</td>
<td>ribs</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>-1.17</td>
<td>-1.43</td>
</tr>
<tr>
<td>case 6</td>
<td>back</td>
<td>ribs</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>-1.16</td>
<td>-1.09</td>
</tr>
<tr>
<td>case 7</td>
<td>back</td>
<td>ribs</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
<td>-1.30</td>
<td>-1.13</td>
</tr>
<tr>
<td>case 8</td>
<td>back</td>
<td>ribs</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>-1.31</td>
<td>-1.45</td>
</tr>
<tr>
<td>case 9</td>
<td>back</td>
<td>ribs</td>
<td>0.2</td>
<td>0.3</td>
<td>0</td>
<td>-1.19</td>
<td>-1.23</td>
</tr>
<tr>
<td>case 10</td>
<td>back</td>
<td>ribs</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
<td>-1.15</td>
<td>-1.09</td>
</tr>
</tbody>
</table>

*The change rate of energy loss coefficient from Equation S5 in Supporting Information File 1.*

![Figure 15: Influence of (a) height, (b) width, and (c) spacing of microtextures on drag reduction performance.](image)
The DRR alone cannot fully represent the overall energy consumption for the entire impeller system. Thus, we comprehensively consider the two simulation results to guide the microtexture design. The selection criterion is based on achieving the smallest energy loss coefficient and the highest DRR. Following this standard, ribs with $h = 0.2$ mm, $w = 0.3$ mm, and $s = 0.2$ mm (case 8) exhibit the best performance. The maximum DRR and $\eta_\xi$ are $-1.31\%$ and $-1.45\%$, respectively, in case 8, and the drag reduction effect is significant for the whole impeller system with 45 blades.

**Drag reduction mechanism analysis**

The effective method to reduce drag in the flow field is to delay boundary layer separation and inhibit turbulence generation [33]. Because turbulence generation leads to energy dissipation, increasing the energy loss. Therefore, the drag reduction of the microtextured blade surface was analyzed by considering turbulent kinetic energy, eddy viscosity ratio, and flow field.

Figure 16a compares smooth blades and textured blades (case 8) regarding the turbulence in the surrounding flow field. The presence of the microtexture on the blade surface results in a decrease in turbulent kinetic energy at the back end of the blade, thereby reducing energy losses.

Figure 16b compares the eddy viscosity ratio, representing the stress generated by turbulent motion. The microtexture significantly reduces the stress generated by turbulent motion. As a result, the energy loss in the entire flow channel system is substantially reduced.

The influence of the microtexture on turbulent vortices is shown in Figure 17a; the contour shows the pressure distribution in

![Figure 16: The effect of microtexture on (a) turbulent kinetic energy and (b) eddy viscosity ratio around blades. Images used courtesy of ANSYS, Inc.](image1)

![Figure 17: The effect of microtexture on (a) turbulent vortex and (b) overall shear stress distribution. Figure 17a used courtesy of ANSYS, Inc.](image2)
the flow domain. The periodic boundary was used in the two pictures as the streamline release entrance, with identical streamline. It can be seen from the streamline that the microtexture effectively inhibits turbulence generation and reduces system energy consumption. Weakening of turbulences will cause a reduction of wall shear stress, which is reflected in the reduction of friction resistance.

The shear stress distribution on the smooth blade and the microtextured blade is shown in Figure 17b; the blue mark indicates that the placement of microtextures does not change the overall shear stress distribution of the blade. Instead, it generates shear stress fluctuations within the microtextured area.

Wind tunnel experiment with the microtextured blade surface
Surface quality analysis of the microtextured blade

The processed blade, composed of 7075 series aluminum alloy, is displayed in Figure 18. The blade surface quality was assessed using a JSZ6S trinocular stereo microscope; the results showed that the processed blade has high quality and no obvious defects. In order to further analyze the processing quality, a HIROX KH-7700 three-dimensional video microscope was used to examine the microtexture and blade surface morphology, as shown in Figure 19.

The rib surface morphology and dimensional data are shown in Figure 19a. The theoretical height is 0.2 mm, the width is 0.3 mm, the spacing is 0.2 mm, and the rib–tip spacing is 0.5 mm. In contrast, the actual height measures are 0.202 mm, and the actual rib–tip spacing is 0.534 mm. The minimal machining error is due to the utilization of a ball end milling tool with a diameter of 0.2 mm, which has a processing residue at the corner of the bottom rib area (Figure 20a). Figure 19b indicates slight height fluctuations on the surface of the smooth blade, reaching a maximum deviation of 0.007 mm. This can be attributed to the point contact nature of the ball tip tool during the machining process and the spacing between tool paths. Thus, the machining coverage rate does not reach 100%, resulting in a residual height h as shown in Figure 20b. To sum up, the microtexture here meets the quality requirements.

Results of the experiments

The experimental results are given in Table 11 and Table 12. The results in Table 11 indicate that $\xi_0$ rises with increasing angle of attack. The simulation reveals distinct phenomena occurring at three angles of attack; the separation phenomenon and vortex at the back section of the blade become more apparent and intense with the increase of the angle. Table 12 shows that the textured blade has a more significant effect on reducing $\xi_1$ as the flow angle increases. At the flow angle of 57°, $\eta_\xi = -3.7\%$ based on Equation S5 of Supporting Information File 1, which indicates that the microtexture reduces energy consumption and improves the overall aerodynamic performance of the blades.
Figure 19: Microscopy observation of (a) microribs surface morphology and (b) blade surface morphology.

Figure 20: Machining error analysis diagram of (a) the microtexture and (b) plane processing.

Figure 21 and Figure 22 illustrate the distribution of $L_{CTP}$ and outlet Ma at three different angles of attacks. A higher $L_{CTP}$ and a lower Ma indicate a poorer aerodynamic performance of the blade. According to Figure 21a and Figure 22a, the blade with microtexture exhibits an increase in $L_{CTP}$ from 0.4 to 0.5, while the Ma in the flow channel center decreases from 0.2 to 0.13. These results indicate that, at the flow angle of 52.8°, the microtexture has an adverse effect on the aerodynamic performance of the blade, resulting in increased drag. Figure 21c and Figure 22c show that the blade with microtexture yields a smaller $L_{CTP}$ compared to the smooth blade, and the Ma is slightly higher. These results indicate a reduction in system
Table 11: Experimental results at inlet and outlet of the smooth blade.

<table>
<thead>
<tr>
<th>Angle of attack (°)</th>
<th>$T_1$ (Pa)</th>
<th>$P_1$ (Pa)</th>
<th>$T_2$ (Pa)</th>
<th>$P_2$ (Pa)</th>
<th>$V_1$ (m/s)$^a$</th>
<th>$V_2$ (m/s)$^b$</th>
<th>$\xi$ (%)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>52.8</td>
<td>108056</td>
<td>98763</td>
<td>105010</td>
<td>100920</td>
<td>123.66</td>
<td>91.56</td>
<td>41.61</td>
</tr>
<tr>
<td>54.8</td>
<td>109091</td>
<td>98833</td>
<td>104266</td>
<td>101071</td>
<td>129.50</td>
<td>69.00</td>
<td>58.98</td>
</tr>
<tr>
<td>57.0</td>
<td>111402</td>
<td>99905</td>
<td>104647</td>
<td>101292</td>
<td>135.91</td>
<td>70.21</td>
<td>65.45</td>
</tr>
</tbody>
</table>

$^a$inlet velocity; $^b$outlet velocity; $^c$energy loss coefficient of the smooth blade.

Table 12: Experimental results at inlet and outlet of the microtextured blade.

<table>
<thead>
<tr>
<th>Angle of attack (°)</th>
<th>$T_1$ (Pa)</th>
<th>$P_1$ (Pa)</th>
<th>$T_2$ (Pa)</th>
<th>$P_2$ (Pa)</th>
<th>$V_1$ (m/s)$^a$</th>
<th>$V_2$ (m/s)$^b$</th>
<th>$\xi$ (%)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>52.8</td>
<td>108840</td>
<td>99248</td>
<td>105190</td>
<td>101131</td>
<td>125.23</td>
<td>79.23</td>
<td>46.17</td>
</tr>
<tr>
<td>54.8</td>
<td>109810</td>
<td>99393</td>
<td>104571</td>
<td>101269</td>
<td>130.10</td>
<td>69.38</td>
<td>60.09</td>
</tr>
<tr>
<td>57.0</td>
<td>110853</td>
<td>99359</td>
<td>104828</td>
<td>101488</td>
<td>136.24</td>
<td>69.87</td>
<td>63.02</td>
</tr>
</tbody>
</table>

$^a$inlet velocity; $^b$outlet velocity; $^c$energy loss coefficient of the microtextured blade.

Figure 21: Distribution of $LC_{TP}$ of the single flow channel at angles of attack of (a) 52.8°, (b) 54.8°, and (c) 57.0°.

energy loss. Overall, the microtexture arranged in the back section of the blade positively impacts aerodynamic performance and reduces system energy loss, particularly at the angle of attack of 57°.

The microtexture was arranged at the back end of the blade suction surface based on the analysis of the simulation results, and the drag reduction effect of the microtexture was verified in the wind tunnel experiment. As shown in Figure 23, the drag...
Conclusion

This paper studies an axial flow compressor and presents a simplified numerical simulation method for the rotating blade surface. Furthermore, microtexture design and simulation analysis are carried out on the blade surface to explore the drag reduction performance and mechanism of microtexture. The conclusions are as follows: (1) A simplified simulation method is proposed from the whole impeller to a single impeller blade, establishing the relationship between plane and surface. Theoretical calculations and numerical simulations are employed to design and verify the optimal microtexture for drag reduction performance. The determined microtexture dimensions are a height of 0.2 mm, a width of 0.3 mm, and a spacing of 0.2 mm. (2) The drag reduction mechanism is analyzed and compared for microtextures with different geometric size factors. The presence of microtextures on the blade surface effectively impedes turbulence generation, thus, reducing the turbulent kinetic energy and wall shear stress to reduce drag. (3) The reduction performance of the microtexture blade is the best when the angle of attack is 57°; \( \eta \) in the experiment can reach \(-3.7\%\). Although the difference between the simulation results and the experimental results is large under the other two attack angles, the trend of \( \eta \) of the two results is the same. The larger the angle of attack, the smaller \( \eta \).
simulation results reveal that positioning the optimally sized microstructure at the back end of the blade yields significant benefits. The DRR for a single blade reaches 1.31%, accompanied by a reduction of 1.45% in ηc. (4) A blade cascade experiment is conducted in the high-speed wind tunnel to analyze the energy loss coefficient and wake loss distribution. The results demonstrate a reduction in energy consumption of 3.7% at a flow velocity of 136.24 m/s and an attack angle of 57°.

Bionic microstructures have little influence on the overall strength of the objects they are attached to because of their small size. Their particular functions are of high research value in the application of object surfaces, but there are also some challenges in practical applications. The cost of microstructures in large-area manufacturing and application is large. However, the size effect is the key of microstructures exhibiting good performance. Hence, the large-area manufacturing of high-precision microstructures is worth studying. Chemicals (e.g., polydimethylsiloxane) can quickly replicate biomimetic microstructures, but the operation process is complex, and the soft surfaces are not suitable for surfaces in high-speed flows.

Supporting Information
Supporting information text contains the hydrodynamic theory covered in this paper, including the boundary layer theory, the formulas for calculating the drag reduction performance of the blade and a description of flow separation on the blade surface.

Supporting Information File 1
Boundary layer theory, drag reduction formulas, and blade surface flow.
[https://www.beilstein-journals.org/bjnano/content/supplementary/2190-4286-15-70-S1.pdf]

Author Contributions
Qinsong Zhu: methodology; visualization; writing – original draft; writing – review & editing. Chen Zhang: conceptualization; supervision; writing – review & editing. Fuhang Yu: investigation; methodology. Yan Xu: investigation; resources.

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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.

Preprint
A non-peer-reviewed version of this article has been previously published as a preprint:
https://www.beilstein-journals.org/xiv/download/pdf/20243-pdf

References

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The definitive version of this article is the electronic one which can be found at: https://doi.org/10.3762/bjnano.15.70
Abstract

Many insect species have found their way into ageing research as small and easy-to-keep model organisms. A major sign of ageing is the loss of locomotory functions due to neuronal disorders or tissue wear. Soft and pliable attachment pads on the tarsi of insects adapt to the substrate texture to maximize their real contact area and, thereby, generate attachment during locomotion. In the majority of stick insects, adhesive microstructures covering those pads support attachment. Stick insects do not molt again after reaching the imaginal stage; hence, the cuticle of their pads is subject to continuous ageing. This study aims to quantify how attachment ability changes with age in the stick insect *Sungaya aeta* Hennemann, 2023 and elucidate the age effects on the material and microstructure of the attachment apparatus. Attachment performance (adhesion and friction forces) on substrates with different roughnesses was compared between two different age groups, and the change of attachment performance was monitored extending over a larger time frame. Ageing effects on the morphology of the attachment pads and the autofluorescence of the cuticle were documented using light, scanning electron, and confocal laser scanning microscopy. The results show that both adhesion and friction forces decline with age. Deflation of the pads, scarring of the cuticle, and alteration of the autofluorescence, likely indicating stiffening of the cuticle, were observed to accumulate over time. This would reduce the attachment ability of the insect, as pads lose their pliant properties and cannot properly maintain sufficient contact area with the substrate.

Introduction

Ageing inexorably affects most living organisms, does not exclude insects, and makes different organs or tissues susceptible to wear or fatigue of material [1]. Research on the time-dependent decline of body functions has often been focused on vertebrates, especially mammals, but insects have found their way into ageing research as well [2-4]. They are easy to maintain and have a short lifespan, and changes in their exoskeleton can be easily observed [5]. The process of ageing has been explored most thoroughly in *Drosophila melanogaster* (Meigen, 1830) and other dipterans, often with special regards to flight
Phasmatoidea, also known as stick and leaf insects, are a lineage of large terrestrial insects encompassing around 3500 described species thriving in different habitats [19,20]. They are exclusively herbivorous and camouflage themselves as twigs, leaves, or bark [19,21]. As phasmids are slow and most of them wingless or unable to fly, they adapted strongly to their local environment [11,19,22,23]. Phasmids have evolved considerably depending on plants since pre-angiosperm times [24]. As plants display a huge range of different surface characteristics [23-28], the diversity of microstructures on phasmatoidean attachment pads is assumed to result from adaptations towards these plant surfaces [23,29]. Phasmids possess smooth adhesive pads on their tarsomerers, the euplantulae, and one larger pad at the pretarsus, the arolium [30]. Investigations of the specific functionality of both euplantulae and the arolium by Labonte and Federle [31] have shown that the arolium and euplantulae each perform different tasks. The arolium is used while climbing upside down, whereas the euplantulae generate friction and are used in upright walking. Phasmid euplantulae are covered with different surface microstructures that are likely adapted to specific surface parameters in their environments [32-34]. It has been shown that nubby euplantulae perform better on rough surfaces whereas pads without protrusions perform better on smooth surfaces [35]. Experimental studies concerning the attachment ability of phasmids investigated various functions of this system and how it changes under certain conditions, such as substrate geometry [36], the presence or absence of claws [37], different surface characteristics of substrates [33,38,39], and the combined effect with pad fluids [40]. For these animals, whose lives strongly depend on plants for camouflage and nutrition, attachment to the plant surface is crucial for survival [11,14,21].

Their life history makes phasmids interesting study subjects for ageing research, as this lineage represents some of the largest insects known and species that have a prolonged life expectancy of up to three years after imaginal molt [41]. After this last molt, phasmids do not molt anymore and, hence, their cuticle is subject to continuous ageing. So far, representatives of Phasmatoidea and their adhesive systems have not been investigated with regards to ageing. Nevertheless, the stiffness of the cuticle of these organs and the internal pressure are important for the functionality and likely susceptible to decay during ageing [42,43]. We investigated the change in attachment ability and tarsal morphology in the species Sungaya aeta Hennemann, 2023 (Heteropterygidae). Members of Heteropterygidae can reach impressive life expectancies [41,44], with anecdotal reports extending over five years. The change in attachment performance was quantified through attachment force measurements. Because of the different properties of arolium and euplantulae [31,33], the attachment forces of whole animals were compared in two directions. The pull-off force was measured perpendicular to the substrate, and the traction force parallel to the substrate, to assess the ability of the insect to attach itself in the respective direction and evaluate potential differences arising from performance decay of either of the two components of the overall attachment system.

The aim of this study was to answer the following research questions: (1) Does the attachment ability of older animals differ from that of younger animals? (2) Do pull-off and traction forces on the same substrates change during age? (3) Does the morphology of the tarsus and the attachment pads differ between younger and older animals?

Materials and Methods

1 Animals and experimental conditions

Two groups of 15 adult females per group of Sungaya aeta Hennemann, 2023 (Phasmatoidea: Heteropterygidae, Figure 1)
were selected from laboratory stock (Department for Functional Morphology and Biomechanics, Kiel University), kept under ambient conditions, and fed with fresh blackberry leaves ad libitum. This species was previously referred to as *Sungaya inexpectata* Zompro, 1996, until the original population of this widespread culture stock from Bataan Province, Iliin Forest, Philippines was described as a new species [45]. The groups were selected by age, that is, “younger” females molted into the adult stage about 1 month before experiments started and “older” females ca. 3.5–4.0 months after molt respectively. The age difference between groups was approximately ten weeks. Animals were only considered for experiments with all legs and tarsi completely intact. Prior to the measurements, animals were weighed using a precision scale (Mettler Toledo AG204 DeltaRange, Mettler-Toledo International Inc., USA). Measurements were conducted during daytime, at a temperature of 24.6 ± 1.9 °C and an ambient humidity of 51.0% ± 6.9%. Deceased animals were frozen at −70 °C for subsequent investigation of the tarsal morphology.

2 Attachment on a smooth incline
The adhesive abilities on a smooth incline were determined using a custom-made tilting platform following the methodology of Berthé et al. [46] with a glass plate as substrate for attachment. Each animal was placed onto the horizontal glass plate, and the plate was then slowly tilted with an average angular velocity of ca. 3.5° per second until the insect started to slide down or fell off (Figure 2A). The positions and orientations of the animals were standardized, that is, always in the center of the plate with the head facing in the same direction. Values were recorded in intervals of 5°, and the mean of the three measurements was considered for further analysis.

3 Force measurements
Attachment force measurements were conducted using a BIOPAC MP100 data acquisition system with a TCI-102 interface (BIOPAC Systems, Inc., USA) and a 100g force transducer (Fort100, World Precision Instruments, Sarasota, FL) using the setup described in Winand and coworkers [37]. We measured pull-off (perpendicular to the substrate) and traction (parallel to the substrate) forces on substrates with three different roughnesses. The substrates were fixed onto a scissor lab jack or a precision slide (Cleveland Lineartecnik GmbH, Löfflingen, Germany). We used glass and epoxy resin [47] replicas of substrates with 1 µm and 12 µm roughness according to Büscher and Gorb [33] to test for differences in the attachment performance on different degrees of surface roughness during ageing. This range of substrate roughness was selected to test for different aspects of the functionality of the attachment pads without major influences of the claws [33,37].
On smooth surfaces (0 µm), smooth pads generate proper contact with the surface. Microrough surfaces interfere with the contact formation of smooth pads; however, the dimension of the nubs on the euplantulae yield different responses to the roughness because fine roughness (1 µm) matches the size of the tips and course roughness (12 µm) matches the size of the entire nubs [33]. The combination of these three levels of roughness was used to investigate potential effects in the three mentioned perspectives of the attachment pads. For details on the fabrication process, the roughness parameters, and the contact angles of the substrates, see [12]. Per trial, the respective force was measured three times per animal and substrate. The order of the substrates was randomized for each direction (pull-off and traction forces) and animal. The insect was anaesthetized with carbon dioxide for 10–15 s before being connected to the force transducer by a string of fishing line (0.18 mm) at the mesothorax at the estimated center of mass [33,48]. The animals were allowed to recover for some minutes until they were responding to being lifted off the substrate with leg movements.

Time–force curves were recorded using AcqKnowledge (3.7.0, BIOPAC Systems, Inc., USA) while moving the substrate and platform manually with steady speed in the required direction. The maximum traction or pull-off force was recorded (see [33]). The means of the three measurements per trial were used for data analysis to reduce intra-individual variance. As one old female deceased within the experimental time, the sample size was 15 for both groups regarding pull-off forces and for the young group regarding traction forces; the sample size was 14 for traction force measurements in the old group. A list of all measurements of attachment forces and body weights is included in Supporting Information File 1.

4 Attachment over time
To further investigate the relation between progressing age and pull-off/traction force performance, six of the younger animals were used for further experiments. The abovementioned attachment measurements (see sections 2 and 3) were repeated once a week for six consecutive weeks. The measurements started ca. 1.5 months post adult molt. The order of substrates and the direction to be measured first were randomized per animal and week.

5 Light microscopy
The tarsi of all animals were documented postmortem using a stereo microscope (Nikon SMZ745T, Nikon Corporation, Tokyo, Japan). Pictures were taken using a Sony DSC-RX0 (Sony Group Corporation, Tokyo, Japan) equipped with a C-mount adapter using a RX0 Mod Kit (Back-Bone Gear Inc., Ontario, Canada). Frozen animals were allowed to thaw, and tarsi were removed for examination. Stacks of images were taken from different focus planes and combined subsequently. Images were processed using Adobe Photoshop v24.7 and Adobe Lightroom Classic 12.0 (Adobe Inc., San Jose, USA). After focus stacking and cropping, clarity and contrast were adjusted.

6 Widefield fluorescence microscopy (WFM)
Autofluorescence signals of insect cuticle can be used to investigate the material composition of the arthropod exoskeleton [49]. To scan for differences in the fluorescence, a selection of
Tarsi across all age groups were examined using WFM. Freshly molted adult and subadult individuals were acquired from laboratory stock and used for imaging as well. Three individuals were chosen for each age group.

Tarsi were cut off at the tarso-tibial joint and transferred into 1.5 mL solution of phosphate-buffered saline (PBS) and Triton TM-X100 (Sigma-Aldrich, St. Louis, USA) for 30 min to reduce surface tension and enable proper glycerin coating. Afterwards, samples were rinsed three times in glycerin and then fully submerged in glycerin and covered with a high-precision cover slip (Carl Zeiss Microscopy GmbH, Jena, Germany).

Images were taken using a Zeiss Axiosplan microscope and an AxioCam MRc camera with the AxioVision software (v. 4.8.2) (Carl Zeiss AG, Oberkochen, Germany). The tarsi were examined at 5× magnification. Sets of excitation and emission filters were used according to [50].

7 Confocal laser scanning microscopy (CLSM)

A confocal laser scanning microscope (Zeiss LSM 700, Carl Zeiss Microscopy GmbH, Jena, Germany) with stable solid-state lasers (wavelengths 405, 488, 555, and 639 nm) and the corresponding band- and longpass emission filters (BP 420–480, LP490, LP560, and LP640 nm) was used to obtain detailed information about the autofluorescence of the cuticle [50]. The samples were prepared the same way as for WFM imaging (see section 6). One tarsus per respective age group was examined (subadult, freshly molted adult, young, and old). The ZEN2008 software (Carl Zeiss AG, Oberkochen, Germany) was used to generate maximum intensity projections.

8 Scanning Electron Microscopy (SEM)

For inspection of the tarsal morphology of different age groups, samples were chosen after CLSM to compare regions of interest, such as altered autofluorescence or damage. Selected tarsi were transferred from glycerin into 50% ethanol via a gradual series of glycerin (descending) and ethanol (ascending) mixtures. Afterwards, samples were dehydrated in an ascending ethanol series and dried using a Leica EM CPD300 (Leica, Wetzlar, Germany) critical point drier. The tarsi were mounted on SEM stubs and sputter-coated with 10 nm gold–palladium in a Leica Bal-TEC SCD500 (Leica Camera AG, Wetzlar, Germany) coater. A Hitachi TM3000 (Hitachi Ltd. Corporation, Tokyo, Japan) scanning electron microscope was used to document the tarsal morphology at 15 kV acceleration voltage.

9 Data analysis

Data analysis was performed in the R environment [51] using R Studio [52]. Data was tested for normal distribution and homoscedasticity using Shapiro–Wilk test and Levene’s test, respectively, the latter from the “car” package [53]. Performance by direction and substrate for time series over six weeks was compared with One Way Repeated Measures Analyses of Variance (ANOVA) and Tukey’s post-hoc test or Friedman’s Repeated Measures ANOVA and Dunn’s post-hoc test with Holm correction (“FSA” package, [54]), depending on the results of the preassumption tests. For pull-off and traction forces of old and young animals, Kruskal–Wallis One Way ANOVA and Dunn’s test with Holm correction were used instead. Wilcoxon rank sum test was used for the comparison of sliding angles between old and young adult animals, according to the results of the Shapiro–Wilk test.

Results

Attachment on a smooth incline

On the tilting platform, young adult animals started sliding or detached from the substrate at 179.87° ± 0.52° (mean ± SD), whereas older animals lost grip at 118.87° ± 54.98° (Figure 2B). Instances where angles of 180° were reached did not cause the animals to slide. Despite the amount of variation among sliding angles on glass in older animals (range: old = 123.33°, young = 1.67°), the sliding angles of young adult animals were significantly higher than those of old adult animals (Wilcoxon rank sum test, $U = 40.500$, $p < 0.001$).

The attachment ability of the younger adult animals ($N = 6$) that were tested over the range of six consecutive weeks faded gradually (Figure 2C). The maximum angle at which the animals started sliding off the incline declined significantly (One Way Repeated Measures ANOVA, $F = 12.299$, d.f. = 5, $p < 0.001$). In the first three weeks, the mean sliding angle decreased slowly (week 1: 180.0° ± 0.0°; week 2: 168.6° ± 20.4°; week 3: 154.2° ± 36.0°). The mean sliding angles in these three weeks did not differ significantly from each other (Dunn’s test with Holm correction, all $p > 0.050$). The mean sliding angle dropped to 110.0° ± 46.4° in week 4, which was significantly lower compared to the first two weeks (Dunn’s test with Holm correction, all $p < 0.05$), but not different from week 3 (Dunn’s test with Holm correction, $p = 0.092$). The sliding angles further decreased in week 5 (82.3° ± 41.69°) and week 6 (86.5° ± 37.5°), resulting in significantly lower sliding angles compared to the weeks 1–3 (Dunn’s test with Holm correction, all $p < 0.050$). From week 5 to week 6, sliding angles remained similar (Dunn’s test with Holm correction, $p = 0.752$). Variance increased over time.

Attachment forces

Attachment performance of young and old animals

In the pull-off direction (Figure 3A), both age and substrate had some effect on the measured forces (Kruskal–Wallis ANOVA...
Figure 3: Attachment force measurements. (A, B) Comparisons of attachment forces of old and young adult females on substrates with different roughness. (A) Pull-off forces and (B) traction forces. * = \( p < 0.05 \), Kruskal–Wallis ANOVA on ranks/Dunn’s post-hoc test with Holm correction.

(C, D) Change of attachment forces of adult females on substrates with different roughness over the course of six weeks. Colors in C and D represent the same substrate as indicated on the top of the graph. (C) Pull-off forces. (D) Traction forces. * = \( p < 0.05 \), Kruskal–Wallis ANOVA on ranks/Dunn’s post-hoc test with Holm correction. Boxes cover the interquartile range (IQR) from 25th to 75th percentile, the black line indicates the median. Whiskers extend to 1.5·IQR.

on ranks, \( H = 66.677 \), d.f. = 5, \( N_{\text{young}} = 15 \), \( N_{\text{old}} = 15 \), \( p \leq 0.001 \). In young adult animals, pull-off forces differed significantly between the three substrates (Dunn’s test with Holm correction, all \( p < 0.010 \)) and were highest on glass (124.38 ± 22.55 mN), less high on 1 µm (75.48 ± 13.51 mN), and lowest on 12 µm rough substrates (54.85 ± 8.72 mN). No significant effect was found between substrates in older animals (Dunn’s test with Holm correction, all \( p > 0.050 \)). The pull-off forces for old animals were also highest on glass (50.37 ± 21.46 mN), less high on 1 µm (34.09 ± 8.48 mN), and lowest on 12 µm rough substrates (32.46 ± 4.59 mN). Younger animals performed significantly better on all substrates compared to older animals on the same substrate (Dunn’s test with Holm correction, all \( p < 0.050 \)).

Traction forces (Figure 3B) showed relationships qualitatively similar in different animals to pull-off forces. In younger adult animals, traction forces were significantly influenced by the substrate roughness (Kruskal–Wallis ANOVA on ranks, \( H = 72.314 \), d.f. = 5, \( N_{\text{young}} = 15 \), \( N_{\text{old}} = 14 \), \( p \leq 0.001 \)). Similar to the pull-off forces, the highest values were obtained on glass (122.31 ± 36.48 mN), lower forces on 1 µm (82.38 ± 15.25 mN), and lowest on 12 µm rough substrates (66.78 ± 14.8 mN). The traction forces on the three substrates differed significantly from each other in young adult animals (Dunn’s test with Holm correction, all \( p < 0.050 \)). Traction forces of older animals were influenced by the substrate as well (Kruskal–Wallis ANOVA on ranks, \( H = 72.314 \), d.f. = 5, \( N_{\text{young}} = 15 \), \( N_{\text{old}} = 14 \), \( p \leq 0.001 \)). The forces were significantly higher on glass (43.13 ± 14.2 mN) compared to 1 µm (21.48 ± 14.26 mN) and 12 µm rough substrates (22.93 ± 8.74 mN) (Dunn’s test with Holm correction, all \( p < 0.050 \)). No significant difference was found between 1 and 12 µm rough substrates (Dunn’s test with Holm correction, all \( p > 0.050 \)). Differences between age groups on the same substrate were all significant (Dunn’s test with Holm correction, all \( p < 0.050 \)).

Signs of ageing were apparent during the attachment force measurements. Older animals were observed to establish less rigorous contact of their tarsi with the substrates at some occa-
sions. During traction force measurements, sometimes tarsi were not aligned with the direction of the pulling movement and were sliding more easily compared to other tarsi. However, these problems with contact formation were not persistent throughout the experiments and occurred only from time to time.

**Attachment forces over time**

Variances of pull-off forces were higher on glass and 1 µm roughness during the first weeks and decreased towards the fifth and sixth week, whereas results on 12 µm roughness showed the least variance across the time span. All three substrates revealed significant differences over time (RM ANOVAs, all \( p \leq 0.001 \)). The pull-off force on glass (RM ANOVA, \( F = 22.437, \text{d.f.} = 5, p \leq 0.001 \)) gradually decreased from 112.34 ± 24.83 mN in week 1 to 29.790 ± 0.56 mN in week 6. The changes of pull-off force on glass between week 1 and weeks 3–6 (Tukey’s tests, all \( p < 0.005 \)), between week 2 and weeks 4–6 (Tukey’s tests, all \( p < 0.001 \)), and between week 3 and weeks 5–6 (Tukey’s tests, all \( p < 0.030 \)) were found to be significant. On 1 µm roughness (RM ANOVA, \( F = 14.346, \text{d.f.} = 5, p \leq 0.001 \)), the forces were lower than on glass in week 1 (77.72 ± 22.11 mN) and declined to 23.72 ± 4.49 mN in week 6. The changes of pull-off force on 1 µm between week 1 and weeks 3–6 (Tukey’s tests, all \( p < 0.003 \)) as well as between week 2 and weeks 4–6 (Tukey’s tests, all \( p < 0.001 \)), and between week 3 and weeks 5–6 (Tukey’s tests, all \( p < 0.001 \)) differed significantly. On 12 µm (RM ANOVA, \( F = 15.618, \text{d.f.} = 5, p \leq 0.001 \)), the forces were lower than on glass in week 1 (53.88 ± 8.21 mN) but still decreased towards week 6 (28.86 ± 9.83 mN). The changes from week 1 to weeks 4–6 (Tukey’s tests, all \( p < 0.018 \)) as well as from week 2 to weeks 4–6 (Tukey’s tests, all \( p < 0.039 \)) were significant as well. The pull-off forces of the remaining combinations did not differ significantly from each other (Tukey’s tests, all \( p > 0.15 \)).

The mean traction forces declined on all surfaces over time following the same trends as the pull-of forces (Figure 3D). The traction changed significantly over time as well (RM ANOVAs, \( F_{\text{glass}} = 16.484, F_{1 \mu m} = 12.540, F_{12 \mu m} = 8.784, \text{d.f.} = 5, \text{all } p \leq 0.001 \)). Forces declined from 126.90 ± 54.18 mN (glass), \( 85.99 \pm 23.27 \text{ mN (1 µm), and 69.84 ± 24.59 mN (12 µm) to 35.60 ± 1.52 mN (1 µm), and 28.58 ± 3.47 mN (12 µm). For glass, the changes between week 1 and weeks 4–6 (Tukey’s tests, all \( p < 0.003 \)), between week 2 and weeks 4–6 (Tukey’s tests, all \( p < 0.007 \)), and between week 3 and weeks 5–6 (Tukey’s tests, all \( p < 0.003 \)) were significant. On 1 µm roughness, forces changed significantly from week 1 to weeks 4–6 (Tukey’s tests, all \( p < 0.011 \)), from week 2 to weeks 5–6 (Tukey’s tests, all \( p < 0.002 \)), and from week 3 to weeks 5–6 (Tukey’s tests, all \( p < 0.008 \)). On the 12 µm substrate, only changes from week 1 to weeks 4–6 (Tukey’s tests, all \( p < 0.360 \)) and from week 2 to weeks 4–6 (Tukey’s tests, all \( p < 0.044 \)) were significant. The traction forces of the remaining combinations did not differ significantly from each other (Tukey’s tests, all \( p > 0.06 \)).

**Morphological changes**

**Macroscopic changes of attachment devices**

All tarsi of *S. aeta* possess five euplantulae on their five tarsomeres and one arrolium situated between two claws on the preatarsus (Figure 1). Ageing was mainly visible from the shape of the attachment pads themselves (Figure 4). Observations via stereomicroscopy showed that in younger animals all attachment pads are fully inflated and appear tightly filled with the fluid (Figure 4A). The condition of the attachment pads varied in older animals. Euplantulae and arolia were frequently observed to be sunken in or shriveled and discolored (Figure 4B–D). Additionally, the same pads showed variance in deflation across different specimens or legs of the same animal. Also, the attachment pads differed in the degree of deflation, depending on the tarsal segment they are located on. The degree of deflation of the pads was always higher in the distal ones. The distalmost arrolium was most strongly affected by deflation in most of the cases (Figure 4B–D), whereas the degree of deflation in euplantulae differed depending on how distal the particular euplantula was situated on the tarsus (Figure 4C,D). Overall, the extent of deflation varied across the specimens and tarsi of the same animal. However, the deflation was generally strongest for older animals.

Claws were uniform in color, but claw wear was observed to vary across specimens (Figure 4E–H). In general, claw conditions ranged from fully intact (Figure 4E) to completely missing (Figure 4H). Most frequently the claw tips were broken (Figure 4F,G). The wear was strongest in older animals, but observed through all age groups.

**Material changes**

Changes of the cuticle of the attachment pads were investigated via WFM and CLSM. Both methods were used to visualize the autofluorescence of the pad cuticle, which is informative about the cuticle composition, for example, the degree of sclerotization [50,55]. Both methods indicate the degree of sclerotization through the autofluorescence of the materials excited with light of different wavelengths. The detected autofluorescence signals are visualized in different colors according to the excitation wavelength [50]. Blue indicates less sclerotized cuticle, green indicates rather sclerotized cuticle, and red colors indicate strongly sclerotized cuticle [50,55]. The general appearance of the autofluorescence and its distribution was uniform for all tarsi examined and corresponds to the signals known for stick
insect tarsi [56]. Differences in color between the pads and the cuticle of the tarsus were clearly visible. The adhesive pads were generally fluorescing blue in both measurements (Figure 5 and Figure 6). Using WFM, the cuticle of the tarsomeres appeared in a yellow-orange color (Figure 5) and showed red and green signals in CLSM (Figure 6), indicating their stronger degree of sclerotization. A double row of dots with red autofluorescence located on the pads along the central groove was visible using WMF (e.g., Figure 5C); it can be assigned to the position of mechanoreceptors (see Figure 7D,H below and also [37]). No differences in the autofluorescence pattern were seen among front, middle, and hind legs.

The blue color of the cuticle of attachment pads appeared more vibrant using WFM in the subadult individuals (Figure 6A–D) and young adult animals (Figure 6E–H) than in the older animals (Figure 6I–K). Because of individual settings for each scan of the CLSM, the colors of the maximum intensity projects are not directly comparable among the images. However, the relative distribution of signals can be informative for the comparison of signs of ageing in combination with shape changes of the attachment pads. The deflation of the eupantulae and arolia of older animals is also visible in WFM (Figure 5LI) and CLSM (Figure 6C–E). The deflation leads to strongly wrinkled pad surfaces. The tarsi of young adult animals sometimes revealed smaller patches with derived autofluorescence signals on the attachment pads (Figure 5E). Instead of the vibrant blue signal of the surrounding cuticle, some areas appear orange to brown in WMF (Figure 5F) images, or green to red in CLSM (Figure 6B) images, typical for stronger sclero-
Figure 5: Ventral views of attachment pads obtained from WFM. (A–D) Subadult female. (E–H) Young adult female. (I–L) Old adult female. Images within one row are from different areas of the same sample. Vibrant blue color indicates soft cuticle, dark yellow-red color indicates stiffened cuticle. (A, E, I) Arolia. (B, F, J) Fifth euplantula. (C, G, K) Third euplantula. (D, H, L) First euplantula. Attachment pads show increasing stiffened areas with age and relatively less strong autofluorescence signals of the soft cuticle. Scale bars: 200 µm.

tized cuticle. The size and proportion of such patches was higher on the pads of older animals (Figure 5 I), and large parts of the euplantular area frequently showed an overall reddish hue throughout the pad surface (Figure 5I–L).

Microscopic ageing signs
Several further microscopic signs of ageing were visible using SEM (Figure 7). Wrinkles due to deflation of the pads often caused furrows on the surface of arolia (Figure 7A). While the original condition (Figure 7D) of the euplantula exhibits a bilobed inflated pad without major markings, except for the central groove and the nubby attachment microstructure, different wear marks were observed on the euplantulae of older animals. The wear patterns included scarred scratches (Figure 7E), scarred tissue from larger wounds (Figure 7F), and deformations of the pad surface that potentially arose from
inhomogeneous changes of the material properties of the cuticle (Figure 7G). Other wear marks were found on the claws (Figure 7C) and on the mechanoreceptors of euplantulae (Figure 7I). While the contact sensilla on the euplantulae are usually found in pairs within groves without micro-ornamentation and are well recognizable (Figure 7H), the setae of the mechanoreceptors were often worn off in older animals (Figure 7I).

Certain changes of the attachment pad cuticle that were not visible using some methods were verified with other microscopy techniques (Figure 8). Larger deformations of the attachment pads, visible by stereomicroscopy (Figure 8A), often appeared dark and brownish in WFM (Figure 8C), which could also be due to contamination. SEM revealed most of such cases as not being caused by contaminations. They rather arose from a strong alteration of the cuticle (Figure 8E), also including changes of the surface topography of the terminal layer of the attachment pad cuticle. Profound hardening of the cuticle could yield an appearance similar to a pad coverage by other substances resulting in dark patches in WFM (Figure 8B,H,K). Such patches usually showed no covering films visible in stere-
Discussion

Decay of attachment performance

Older animals showed a decline in adhesive performance similar to cockroaches as previously shown by Ridgel and coworkers [16]. The decline of attachment performance was also measurable here in repetitive tests over a longer time span. Animals lost adhesive abilities on all substrates over the course of six weeks, and their attachment performance converged to the level of the older animals in the first series of experiments. Variation of the attachment performance increased with age, which could be seen in the direct comparison of sliding angles and in the measurements over time. Ageing is a gradual process; hence, the decline of attachment abilities can be expected to be gradual as well and to show intraspecific variation [17]. A difference in activity of the animals was also noticeable during the experiments. The young individuals were more active, whereas the old animals took longer to recover from anesthesia (not quantified). Ridgel and Ritzmann [5] also detected a decrease of around 50% in walking speed of aged cockroaches. This matches the proposed loss of muscle fibers with age, leading to muscle atrophy [16].

Roughness dependence of attachment performance decay

The performance of insect attachment pads recorded on a smooth surface is usually higher than on microrough surfaces
Figure 8: Combination of visualizations of the same attachment organs with different microscopy techniques. A–F, G–I, J–K, and L–M correspond to the same respective tarsi of old adult females. Arrowheads mark areas of concern. (A, G) Stereomicroscopy images showing the appearance of the attachment pads. (B, C, H, K, M) WMF images showing native bluish regions and those stiffened due to the ageing. (D–F, I, J, K) SEM images showing the topography of the surface. Scale bars: (A) 1 mm, (B–M) 500 µm.
In contrast to insects; this was shown to enable regeneration of the properties of the integument of the attachment pads. Geckos, that is, damage, contaminations, and changes of material face similar challenges to sustain attachment during ageing different roughnesses, depending on the morphology of their adhesion ability, no safety factors (attachment force per weight force) below 1 were observed, even in older animals; according to Pillai et al. [62], this would have been an indicator for the failure of the adhesive system to statically hold the insect’s weight on the ceiling. Apart from insects, roughness plays a role in adhesion of non-arthropods as well. Roughnesses of 100–300 nm had the largest attachment-reducing effect for both single setae and whole geckos in experiments with the species S. aeta [31]. Apparently, the ratio of the nubs, compared to the surface asperities, makes better contact on a 1 µm rough surface, if compared to a 12 µm rough surface in younger adult individuals. This effect vanished for older adult animals, as attachment forces became more similar on all three substrates. No change of nub morphology was observed in older animals (Figure 7G, I), but a change of stiffness of the nubs could potentially affect their functionality as well. Nevertheless, pad compliance and deflation likely have a stronger effect in this experiment as discussed below. Other studies reported lowest forces on 1 µm and higher forces on 12 µm roughness for adhesion and traction in other arthropods [59, 60]. This effect was presumably due to differences in micromorphology of their adhesive systems. Despite the gradual decay of attachment ability, no safety factors (attachment force per weight force) below 1 were observed, even in older animals; according to Pillai et al. [62], this would have been an indicator for the failure of the adhesive system to statically hold the insect’s weight on the ceiling. Apart from insects, roughness plays a role in adhesion of non-arthropods as well. Roughnesses of 100–300 nm had the largest attachment-reducing effect for both single setae and whole geckos in experiments with the species Gekko gecko (Linnaeus, 1758) [63]. Similar to insects, attachment performance in geckos can vary between species on different roughnesses, depending on the morphology of their adhesive systems [64-67]. Similar to the results shown here, geckos face similar challenges to sustain attachment during ageing [64], that is, damage, contaminations, and changes of material properties of the integument of the attachment pads. Geckos, however, continuously shed their skin throughout their life, in contrast to insects; this was shown to enable regeneration of the adhesive properties of the attachment system to some extent [62].

Claw wear

Claws did not play a role in our attachment experiments, but they were morphologically investigated as part of the tarsus as well. No link between age and claw wear could be established as claw wear usually happens abruptly at single instances and accumulates over time [68]. Damage on the claws consequently rather indicates whether a particular individual claw experienced sufficient stress to be damaged than informs about the age, besides the fact that the longer life time potentially leads to the higher probability of such events. Claws are therefore unsuitable to determine the age of S. aeta. Some arthropod claws, such as those in ticks [69], have been shown to contain small amounts of resilin, an elastomeric protein providing flexibility in cuticle composites [70]. Voigt and Gorb [69] also suspect resilin to occur in other arthropod claws as well, but melanization impedes investigation using fluorescence microscopy. Resilin-containing structures within the claws could potentially work as dazing mechanisms to reduce wear and risk of damage [69]. Claws are presumed to be more resistant to wear than the soft and pliable adhesive pads [68, 71]. Most claw breakages were observed at the tips; the tips have to withstand the greatest stresses, which mostly occur in single events, rather than in normal wear [68]. Further studies could explore the role of fatigue of claw material and its effect on the mechanical properties.

Pad compliance

There are several possible ways in which attachment abilities could be affected by ageing. Compliance of the attachment pad to the substrate plays a significant role for the performance. The compliance of the attachment pad surface can be negatively affected by changes of the material properties of the cuticle and through structural damage of the surface, leading to obstacles for contact formation at the interface between the pad and substrate [11]. Ridgel et al. [16] noticed dry and dark pads in aged cockroaches, but they could not explain why the pads changed appearance and properties. Zhou et al. [17] assumed sclerotized scars to negatively impact pad compliance as such injuries accumulate with age. This effect was also found in different species of tree geckos [62]. A decrease in clinging ability in geckos was recorded with time passed since the last shedding. As claw wear usually happens abruptly at single instances and accumulates over time [68]. Damage on the claws consequently rather indicates whether a particular individual claw experienced sufficient stress to be damaged than informs about the age, besides the fact that the longer life time potentially leads to the higher probability of such events. Claws are therefore unsuitable to determine the age of S. aeta. Some arthropod claws, such as those in ticks [69], have been shown to contain small amounts of resilin, an elastomeric protein providing flexibility in cuticle composites [70]. Voigt and Gorb [69] also suspect resilin to occur in other arthropod claws as well, but melanization impedes investigation using fluorescence microscopy. Resilin-containing structures within the claws could potentially work as dazing mechanisms to reduce wear and risk of damage [69]. Claws are presumed to be more resistant to wear than the soft and pliable adhesive pads [68, 71]. Most claw breakages were observed at the tips; the tips have to withstand the greatest stresses, which mostly occur in single events, rather than in normal wear [68]. Further studies could explore the role of fatigue of claw material and its effect on the mechanical properties.
indicative for changes of the material properties of the cuticle. The autofluorescence correlates with the degree of sclerotization [50,70], and stronger cross-linking usually results in stiffer cuticle. As stiffer cuticle is less compliant, and the lower resulting actual contact area leads to lower attachment performance [72-74]. Most flexible cuticle consists at least partially of resilin [50,75,76], which needs water as a plasticizer to retain its extraordinary mechanical properties [70]. As the water evaporates, resilin becomes brittle and less resilient. Pad cuticle was also found to be more prone to evaporation than the leg cuticle [9], which could amplify the resilin degeneration due to sclerotization. Many of the regions of derived autofluorescence on the arolia and euplantulae did not show structural changes in SEM (Figure 8G–K) and likely represent areas of stiffened pad cuticle. Other ageing marks with derived autofluorescence revealed signs of persistent damage to the pad cuticle (Figure 7 and Figure 8). Such dark spots, observed using both fluorescence techniques, are likely scars resulting from repaired damage of the pad cuticle [17]. Abrasion of attachment pad cuticle can be repaired in insects. If the damage is superficial, epicuticle can be restored by depletion of waxes [18,77], but stronger damage results in sclerotization of the wound due to phenolase activity involved in the wound closure [78]. Such sclerotized scars do not only reduce the stiffness of the repaired area; they also cause structural obstacles that interfere with contact formation and reduce attachment performance [17].

**Pad deflation**

Besides material properties of the cuticle and microscopic surface features, further effects on attachment performance are likely results of the geometry of the attachment pads [43]. In their study, Ridgel and Ritzmann [5] proposed two ways in which age might affect attachment. They assumed either vascular insufficiency or degeneration of the tracheal system to be responsible for cockroach tarsus degeneration. Stiffer and dryer pads might be results of such ageing processes. Tracheal degeneration could lead to leg tissue dying because of a lack of oxygen and, therefore, might also influence the adhesive pads and their performance. The suspected vascular insufficiency ties into the hemolymph pressure. In the insect circulatory system, the low pressure is kept up by the heart and muscular activity [5,79]. The legs function as a terminal end in the circulatory system and often have accessory hearts to enable hemolymph flow [79]. If age has an effect on a stick insect’s cardiac system, any impairment could additionally decrease the initially low hemolymph pressure. The observed stronger effect of ageing on the more distal regions of the tarsus in S. aeta could support the hypothesized influence of lack of the hemolymph support; more distal regions are likely stronger affected by this effect because of their peripheral connection to the circulatory system [79]. A decrease in spontaneous activity has also been reported from senescent insects [16,80]. Less activity would also mean less circulatory support by muscular activity, intensifying the circulatory problems. A relatively large fraction of the tarsus is filled with hemolymph [30], including the volume of the attachment pads [56,81].

In contrast to other insects, such as hymenopterans [82-84], the expansion of the arolium in stick insects is not supported by internal sclerites; instead, it results from the internal pressure within the pad similar to other polyneopteran insects [30,85].

Dening et al. [43] showed that the inflation degree of attachment devices in different animals and artificial attachment devices can play a role in the adhesion control. High pressure within the pad reduced the contact area with the substrate because of the curvature of the pad, and reduced inflation led to larger contact area and increased adhesive performance [43]. As the inflation is achieved by an increased hemolymph pressure in the pads of S. aeta, a decline in hemolymph supply to the pads would reduce inflation. The strong extent of deflation visible in these pads might lead to a decrease in adhesive properties due to the formation of folds in the pad surface, resulting in reduced actual contact area, in contrast to the findings of Dening et al. [43]. Tracheal degeneration could further harm the tarsal organs as a lack of oxygen could lead to damage in tissues. Loss of tissues, for example, exocrine cells within the arolium [81], could potentially also influence fluid production within the arolium. These exocrine cells produce adhesive secretions that play different roles in adhesive systems [11,40,85]. In stick insects, such fluids consist of a watery and a lipid phase [86] and, besides interfacial effects, contribute to the shape and curvature of the terminal layer of the attachment pad [43]. Another factor influencing effective stiffness of the cuticle is caused by depletion of these adhesive fluids. Several steps in quick succession were found to dry out the pad cuticle, making it less flexible and providing reduced attachment [40]. Jiao et al. [73] also reported desiccation and depletion of pad fluid to reduce adhesion in excised tarsi.

Pad deflation could also have negative influence on sensory feedback. The mechanoreceptors on the pads of stick insects, which provide feedback about substrate contact [37], usually occur solely on attachment pads with nubby microstructures and only rarely on smooth eupantulae [34]. The setae of mechanoreceptors are usually mounted in a flexible membrane, which also contains resilin [87]. The combination of a changed pad shape and less flexible membranes surrounding the mechanoreceptors’ setae might impair the function and, therefore, reduce the information the animal is able to receive. In their paper concerning ageing cockroaches, Ridgel et al. [16] propose this lack of
sensory information to negatively impact the ability of old cockroaches to walk up an incline. It seems plausible to assume that the walking speed might also be affected by poor sensory feedback.

**Conclusion**

An effect of age on the attachment abilities of stick insects was found. Attachment and friction forces declined with age on both rough and smooth surfaces. Microscopy investigations revealed deflation of the attachment pads and signs of cuticle hardening, both decreasing pad flexibility and the ability of contact formation to the substrate. The changes observed in the pads of old individuals probably arise from desiccation of the pads and the cuticle, possibly caused by an impaired circulatory system and oxygen deficiency in the tarsus. The effects, such as material desiccation (pads, resilin patches, and membranes), presence of scars on the pad surface, oxygen and hemolymph deprivation, likely reinforce each other. Further experiments could explore more ageing-related effects to gain insights into the processes of attachment ability decay in insects and, thus, potentially improve sustainability of artificial biologically inspired engineering gripping systems. Such studies could include the role of hemolymph pressure for attachment control and the influence of hemolymph within the attachment pads on cuticle hydration and on the production of adhesive fluid.

**Supporting Information**

Supporting Information includes the raw data for all experiments, that is, pull-off forces, traction forces, and attachment angles for all animals, as well as their respective weights.

**Supporting Information File 1**
Raw experimental data. [https://www.beilstein-journals.org/bjnano/content/supporting/2190-4286-15-72-S1.xlsx]

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**Competing Interests**

The authors declare no competing or financial interests.

**Author Contributions**

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

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**Data Availability Statement**

All data that supports the findings of this study is available in the published article and/or the supporting information to this article.

**References**

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