

RESEARCH ARTICLE

Complementary effect of attachment devices in stick insects (Phasmatodea)

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ABSTRACT

Stick insects are well adapted in their locomotion to various surfaces and topographies of natural substrates. Single pad measurements characterised the pretarsal arolia of these insects as shear-sensitive adhesive pads and the tarsal euplantulae as load-sensitive friction pads. Different attachment microstructures on the euplantulae reveal an adaptation of smooth euplantulae to smooth surfaces and nubby euplantulae to a broader range of surface roughness. However, how different attachment pads and claws work in concert and how strong the contribution of different structures is to the overall attachment performance remains unclear. We therefore assessed combinatory effects in the attachment system of two stick insect species with different types of euplantular microstructures by analysing their usage in various posture situations and the performance on different levels of substrate roughness. For comparison, we provide attachment force data of the whole attachment system. The combination of claws, arolia and euplantulae provides mechanical interlocking on rough surfaces, adhesion and friction on smooth surfaces in different directions, and facilitates attachment on different inclines and on a broad range of surface roughness, with the least performance in the range 0.3–1.0 μm . On smooth surfaces, stick insects use arolia always, but employ euplantulae if the body weight can generate load on them (upright, wall). On structured surfaces, claws enable mechanical interlocking at roughnesses higher than 12 μm . On less-structured surfaces, the attachment strength depends on the use of pads and, corroborating earlier studies, favours smooth pads on smooth surfaces, but nubby euplantulae on micro-rough surfaces.

KEY WORDS: Adhesion, Friction, Surface roughness, Biomechanics, Euplantula, Arolium

INTRODUCTION

Stick and leaf insects (Phasmatodea) are distributed worldwide in various environments (Bedford, 1978) and are well known to imitate different parts of the environment, ranging from twigs to leaves, bark and moss (Robertson et al., 2018). Through the realisation of different ecological niches, these insects evolved morphological and behavioural adaptations such as camouflage (Bedford, 1978; Buckley et al., 2008), presence/absence of wings (Whiting et al., 2003), different egg-laying strategies (Carlberg, 1983; Sellick, 1997a,b; Goldberg et al., 2015; Robertson et al., 2018), etc. To cope with the substrate diversity phasmids walk on, they use tarsi consisting of different types of attachment structures, i.e. the pretarsal arolium,


tarsal euplantulae and paired claws. Additionally, euplantulae reveal a high diversity of attachment microstructures (AMS) (Büscher and Gorb, 2017; Büscher et al., 2018a,b, 2019). Adaptations to different environments are even reported for different stages in the post-embryonic development of the same species. For example, *Eurycantha calcarata* dwells in the foliage of shrubs at the nymphal stages (nubby euplantular AMS, developed arolium) and lives on the ground as adults (smooth euplantular AMS, significantly reduced arolium) (Gottardo et al., 2015).

Attachment performance of euplantulae with different AMS was previously studied in representatives of Phasmatodea (Bußhardt et al., 2012) and Orthoptera (Grohmann et al., 2015). Other studies compared the biomechanical properties of arolia and euplantulae on the same tarsus in Phasmatodea (Bennemann et al., 2011; Labonte and Federle, 2014; Labonte et al., 2014). However, studies investigating properties of the pads contributing to adhesion and friction are mostly limited to thorough examination of single compartments of the stick insect attachment system. Previous studies tested friction and adhesion of single euplantulae and arolium separately (Bußhardt et al., 2012; Labonte and Federle, 2014; Labonte et al., 2014). The smooth euplantulae of *Medauroidea extradentata* (in earlier studies misidentified as *Cuniculina impigra* according to Brock et al., 2019) generated stronger adhesion on smooth over rough substrates. The adhesive forces of the structured euplantulae of *Carausius morosus* did not differ between the two substrates. Friction experiments showed anisotropy for both species, with higher values for proximal pulls than for distal pushes at a normal load of 500 μN (Bußhardt et al., 2012). In *M. extradentata*, friction was stronger on the smooth (R_a approximately 0.054 μm) than the rough surface (R_a approximately 1.399 μm) for both directions. However, in *C. morosus*, friction was stronger on the smooth surface only for pushes. This shows that smooth attachment pads are able to generate relatively stronger adhesion and friction on a flat smooth surface than on a rough one. In contrast, nubby pads have similar adhesion on the two substrates, and show no difference to friction in the pulling direction. In *C. morosus*, high adhesion (pull-off force) coupled with shear sensitivity is reported for the smooth arolia and high friction (traction) force coupled with load sensitivity is reported for the nubby euplantulae. This indicates potential specialisation in the use of these pads during locomotion in different behavioural situations (Labonte and Federle, 2014).

The contribution of both friction and adhesion forces, as well as the usage of the different attachment pads in the dynamic process of locomotion, has to the best of our knowledge never been assessed for stick insects. In living animals, both mechanisms obviously work simultaneously and in different situations fulfil their specific functions. In this paper, we therefore studied the performance of the whole attachment system in the animal and compared two species with different euplantular microstructures, i.e. nubby AMS and smooth AMS, regarding the usage of the whole attachment apparatus and its parts on different substrate inclines. We also

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Received 2 July 2019; Accepted 6 November 2019

quantified the contribution of the whole attachment system to both traction and pull-off force generation in animals with different types of AMS on different roughnesses. Specifically, we asked the following questions: (i) how do stick insects use different parts of the tarsal attachment system in different behavioural situations?; (ii) how does the whole attachment system and its parts perform on different degrees of surface roughness?; and (iii) how do both aspects differ between species with smooth or nubby euplantular AMS?

MATERIALS AND METHODS

Specimens

The species were selected according to their euplantular microstructures: *Sungaya inexpectata* Zompro 1996 exhibits a smooth arolium and nubby euplantulae (Büscher et al., 2018a; Büscher et al., 2019), while the euplantulae of *Medauroidea extradentata* (Brunner von Wattenwyl 1907) are smooth. The animals were fed with blackberry leaves *ad libitum* and kept on a regular day/night cycle. Immature individuals of both species were selected from the fifth nymphal stage (L5) for videography and attachment force measurements (see below). All specimens used for this study were obtained from the laboratory cultures of the Department of Functional Morphology and Biomechanics (Kiel University, Germany). Individuals were only used if all six legs were intact.

Scanning electron microscopy

Tarsi or claws were cut off from fifth instar nymphs of both species and fixed in 2.5% glutaraldehyde in PBS buffer for 24 h on ice on a shaker. The samples were then dried in an ascending ethanol series, critical-point dried and sputter coated with gold–palladium to 10 nm thickness. Overview images were taken with a scanning electron microscope (SEM; Hitachi TM3000, Hitachi High-technologies Corp., Tokyo, Japan) at 15 kV acceleration voltage using a rotatable specimen holder (Pohl, 2010). The morphology of the attachment apparatus was observed in the SEM Hitachi S4800 (Hitachi High-technologies Corp.) at an acceleration voltage of 5 kV. Subsequently, the micrographs were processed using Photoshop CS6 software (Adobe Systems Inc., San José, CA, USA). The claw tip diameter (sharpness) and the radius of curvature (half of the tip diameter) were measured from independent specimens ($N=10$ per species) (Dai et al., 2002). All measurements were taken with ImageJ (Schneider et al., 2012).

Videography

To evaluate walking behaviour and attachment pad contact with the substrate, individuals in the fifth post-embryonic stage of both species were selected (mean \pm s.d. body mass: *S. inexpectata* 83.5 \pm 1.9 mg, $N=10$; *M. extradentata* 52.0 \pm 3.2 mg, $N=10$). The animals were filmed while walking on a glass plate in four different situations: (1) walking upright on a horizontal plate, (2) hanging upside down on a horizontal plate, (3) walking up a vertical plate with the animal facing upwards and (4) walking down a vertical plate with the animal facing downwards. We used a Nikon D5300 digital camera (Nikon Corp., Tokyo, Japan) equipped with a macro lens (Canon Macro Lens EF 100 mm, Canon Inc., Tokyo, Japan) and filmed the animals through the glass plate, in order to obtain reliable information about the contact of the attachment pads with the glass. Every individual was filmed in all four situations randomized between the individuals and the situations. The glass plates were carefully cleaned between every sequence with isopropyl alcohol, to avoid any effect of potential residuals of pad fluids of previous individuals. Only sequences of at least 10 steps in the same direction without disruption (turns, falls, etc.) were

analysed and only if the animal walked in a straight line in the direction of the glass plate (i.e. 90 deg from the ground for the vertical plates). The videos were then processed and analysed using the Adobe Premiere Pro CS6 software (Adobe Systems Inc.). We analysed the number of contacts of attachment pads (euplantulae and arolia) with the glass plate for every step of the animal and recorded the walking gait pattern for every sequence. We only noted the general pattern, as described in Grabowska et al. (2012), and did not quantify velocity, phase duration, etc. A pattern was determined if the number of feet in contact was in accordance with the pattern described, with a maximum of 10% of steps deviating from the ground pattern. Sequences observed with more than 10% differing steps, e.g. transitions to a different walking gait pattern, were considered as irregular walking gait patterns.

Force measurements

Specimens of both species (*S. inexpectata* 86.6 \pm 5.6 mg, $N=25$; *M. extradentata* 50.9 \pm 5.2 mg, $N=32$) were attached to a force transducer (25 g capacity; FORT25, World Precision Instruments Inc., Sarasota, FL, USA) by gluing a human hair onto the metanotum of the stick insect with bee wax. The force transducer was connected to a BIOPAC Model MP100 and a BIOPAC TCI-102 system (BIOPAC Systems, Inc., Goleta, CA, USA). Force–time curves were recorded from pulling the animal in the specific direction and visualized with AcqKnowledge 3.7.0 software (BIOPAC Systems Inc.). The highest peak of the obtained graph was considered as the maximum attachment force. For recording the traction force, the animal was pulled backwards along the body axis with a similar setup to that described in Wolff and Gorb (2012a), extended with a motorized micromanipulator (DC 3001R, World Precision Instruments Inc.) to measure passive attachment of the animal. For measuring of the pull-off force, the animal was pulled away from the surface at an angle of 90 deg with the same setup as described by Wohlfart et al. (2014). In both experiments, the sensor was moved by the motorized micromanipulator with a continuous speed of 200 $\mu\text{m s}^{-1}$ and a step size of 10 μm . To investigate performance on different substrate roughnesses, every individual was tested on five different surface replicas in both directions. All surfaces were carefully cleaned with isopropyl alcohol prior to each experiment. As humidity and temperature potentially affect the performance of the attachment pads (Federle et al., 2004; Voigt et al., 2010), experiments were performed at 19–21°C and 20–30% relative humidity. Additionally, the tarsi were filmed during each experiment to document the locomotory behaviour of the insect.

To assess the influence of the claws during attachment, additional specimens (*S. inexpectata* 84.6 \pm 5.9 mg, $N=25$; *M. extradentata* 53.7 \pm 7.4 mg, $N=32$) were anaesthetised with CO₂, all claws were removed with a pair of fine scissors and the specimens were tested with the same experimental setup. Animals were only used if: (1) a sufficient part of the claw was removed to exclude interlocking with the substrate, (2) the residue of the claw did not enable pressure application to the dorsal side of the arolium and (3) no haemolymph leaked out of the tarsus.

Surface preparation

The surfaces used to test traction and adhesion performance were produced using epoxy resin (Spurr, 1969) following the protocol of Salerno et al. (2017). Negative replicas from surfaces of different roughness were obtained using polyvinylsiloxane-based two-component dental wax (Colthène/Whaledent AG, Altstätten, Switzerland). The negatives were filled with epoxy resin and cured at 70°C for 24 h. We used glass polishing papers with a

roughness of 0.3, 1 and 12 μm (Buehler, Lake Bluff, IL, USA) and industrially standardised p40 as templates for the resin replicas. The roughness parameters for all surfaces are provided in Salerno et al. (2017).

Statistical analysis

Statistical analyses were performed using SigmaPlot 12.0 (Systat Software Inc., San José, CA, USA). Prior to subsequent tests, the data were tested for normal distribution (Shapiro–Wilk test) and homoscedasticity (Levene’s test). Traction and pull-off forces in the different postures and species were compared using Kruskal–Wallis one-way analysis of variance (ANOVA) on ranks and Tukey’s *post hoc* test, as the data neither were normally distributed nor showed homoscedasticity. Differences in the attachment forces between the species studied on different roughnesses, as well as differences between specimens of the same species with different treatments (with claws and without claws) on the different roughnesses were compared using the Mann–Whitney rank sum test, if not stated otherwise. For comparisons of two groups that were parametrical and showed homoscedasticity, a *t*-test was performed. We used a chi-square test for comparisons of the walking gait patterns in different species and for comparison of the walking gait patterns in different posture situations, or, if adequate, Fisher’s exact test.

RESULTS

Attachment pad usage

The tarsi of both species studied are similar in structure to those of most other species of stick insects studied and similar to the tarsal morphology of adult individuals of the same species (Büscher et al., 2019). They consist of the usual five tarsomeres with four euplantulae, as well as two claws and one arolium in between (see Fig. 1A–D), typical for Phasmatodea. An accessory euplantula is present on the fifth tarsomere of *S. inexpectata* (Fig. 1A), but not on the fifth tarsomere of *M. extradentata* (Fig. 1B). The AMS of the euplantulae are well documented (Bußhardt et al., 2012; Labonte and Federle, 2014; Labonte et al., 2014; Büscher et al., 2018a,b, 2019) and all are similar to the adults’ nubby euplantulae in *S. inexpectata* (Fig. 1E) and smooth euplantulae in *M. extradentata* (Fig. 1F). In the four different walking situations, (i) upright walking on a horizontal plane, (ii) hanging upside down from a

horizontal plane, (iii) walking up a vertical plane and (iv) walking down a vertical plane, the euplantulae and arolia were employed similarly in the two species (see Movie 1). In all walking situations, the arolium was used for each step (Fig. 2) and was the only attachment pad employed continuously to the same extent. In upright walking, both species employed the two distal-most euplantulae (tarsomeres 3 and 4), in addition to the arolium, in every step independent of the leg (Fig. 2A,E). In *M. extradentata*, the euplantula on tarsomere 2 was occasionally used as well (6–7% of observations in all legs). There were major differences between all legs on the vertical plane (Fig. 2C,D,G,H). While legs above the centre of body mass (forelegs in individuals heading upwards and hindlegs in individuals heading downwards) touched the vertical plane with the arolium only, legs below the centre of body mass also used all four euplantulae (100% for *M. extradentata* and 93–96% for *S. inexpectata*). In general, euplantulae made less contact heading upwards on a vertical plane compared with heading downwards (Fig. 2C,D,G,H). Midlegs mostly used the distal-most two euplantulae when heading upwards and the distal-most three euplantulae when heading down; euplantula 1 (the most proximal one) was rarely used in *S. inexpectata* (6% while heading up and 17% while heading down) and never used in *M. extradentata*. Interspecific differences were observed primarily in the vertical walking situations, where *S. inexpectata* rarely used an additional euplantula in comparison with the other species (Fig. 2C,D,G,H). In horizontal upright walking, *M. extradentata* rarely employed one euplantula more than *S. inexpectata* (Fig. 2A,E). The proportions of euplantula contacts for every step and every posture situation are summarised in Fig. 2.

Based on the videos, we observed three types of hexapedal walking pattern: tripod, tetrapod and wave gait (one leg at a time in swing phase). This is described in detail in previous work (e.g. Grabowska et al., 2012); and one irregular type. The occurrence of these gait patterns differed slightly in the two species studied depending on the orientation of the substrate, except for when animals walked upside down. In this situation, both species applied the wave gait pattern in every sequence recorded (Fig. 3). For both species, the experimental situation and gait pattern revealed a significant relationship (chi-squared test; $\chi^2=14.30$, d.f.=6, $N=10$, $P=0.026$ for *M. extradentata*; $\chi^2=29.57$, d.f.=9, $N=10$, $P<0.001$ for

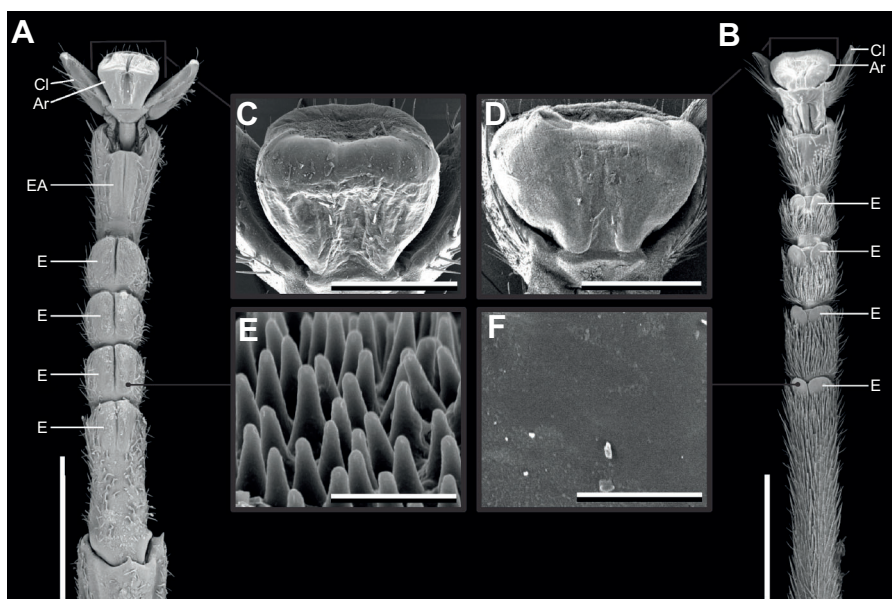


Fig. 1. Scanning electron micrographs of the tarsal morphology of both species.

(A,C,E) *Sungaya inexpectata*, fifth larval stage. (B,D,F) *Medauroidea extradentata*, fifth larval stage. (A,B) Ventral overviews. (C,D) Arolia. (E) Nubby microstructure of the euplantulae of *S. inexpectata*. (F) Smooth microstructure of the euplantulae of *M. extradentata*. Ar, arolium; EA, accessory euplantula (5th euplantula); E, euplantula; Cl, claw. Scale bars: 1 mm (A,B), 300 μm (C,D), 3 μm (E,F).

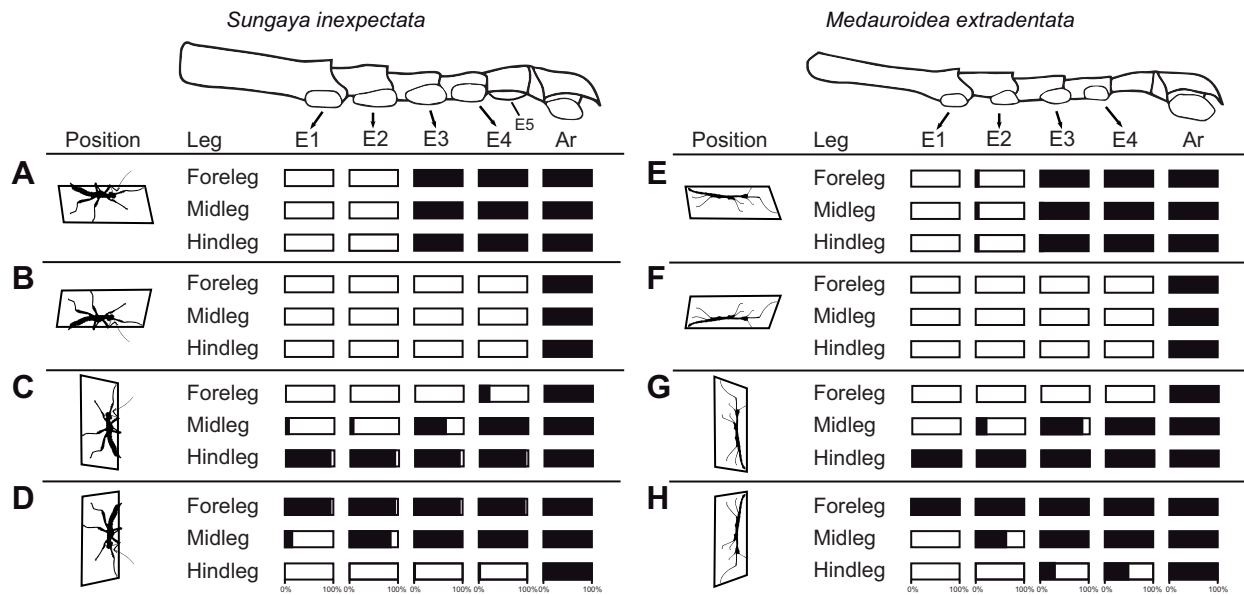


Fig. 2. Percentage of steps in which the particular attachment pad was in contact with the glass plate. (A–D) *Sungaya inexpectata* with nubby euplantular attachment microstructures (AMS). (E–H) *Medauroidea extradentata* with smooth euplantular AMS. (A,E) Animals standing upright on a horizontal plane. (B,F) Animals hanging below a horizontal plane. (C,G) Animals facing up on a vertical plane. (D,H) Animals facing down on a vertical plane. Black shading in the rectangles represents the percentage of observations in which the animals used the particular euplantula.

S. inexpectata). While *M. extradentata* utilized mainly two different gait patterns (tetrapod and wave), *S. inexpectata* very often used the tripod gait in any posture, except when walking upside down. *Sungaya inexpectata* applied the wave gait only while walking up the vertical plane (not down) (20%). In upright walking, the observed gait patterns significantly differed between the species (chi-squared test; $\chi^2=12.21$, d.f.=3, $N=10$, $P=0.007$), with *M. extradentata* applying both the tetrapod (25%) and wave (75%) gaits and *S. inexpectata* mainly using both the tetrapod (50%) and tripod (40%) gaits. In vertical upwards climbing, the occurrence of gait patterns

was independent of the species (chi-squared test; $\chi^2=7.20$, d.f.=3, $N=10$, $P=0.066$). In vertical downwards climbing, the proportion of observations of gait patterns in both species (*M. extradentata* – 100% tetrapod, *S. inexpectata* – 50% tetrapod and 50% tripod) did not vary more than expected from random occurrence (Fisher's exact test, $P=0.101$). Euplantula contact counts of every leg in both species and posture situation are included in Table S1. The fifth euplantula of *S. inexpectata* (Fig. 1A) was not used in any of the examined situations.

Performance of the attachment system

Attachment performance of both species obtained from pull-off and traction measurements is shown in Fig. 4. During our force measurements, the tarsi were used by both species in a similar way. In pull-off experiments, only the arolium remained in contact with the substrate until the pull-off force exceeded adhesion (see Movie 2). The recorded graph shows an increase of pull-off force over time, until the maximum attachment force is reached, and the specimen is completely detached from the substrate (Fig. 4A). During measurements of traction force, animals brought all euplantulae and the arolium into contact with the substrate, and the whole tarsus slid along the surface (see Movie 3). Accordingly, the traction force graphs mostly show more than one local maximum, but several subsequent traction events in which the individual produced a stronger grip. These traction events are separated by phases where the animal slid along the surface, generating a lower amount of traction (see Fig. 4B).

Pull-off forces

General pull-off forces of the whole animals were high on smooth surfaces (about 0 μm roughness; 12.0 ± 4.2 mN) and on p40 (12.4 ± 7.7 mN) for *M. extradentata* (Fig. 4C) and not significantly different from each other (Kruskal–Wallis one-way ANOVA, $H=106.07$, d.f.=4, $N_{1,2}=32$, $P \leq 0.001$; Tukey's test, $P > 0.05$). On 0.3 μm (1.8 ± 0.6 mN) and 1 μm (0.8 ± 0.3 mN) rough surfaces, pull-off forces were significantly lower than on the other surfaces. Pull-

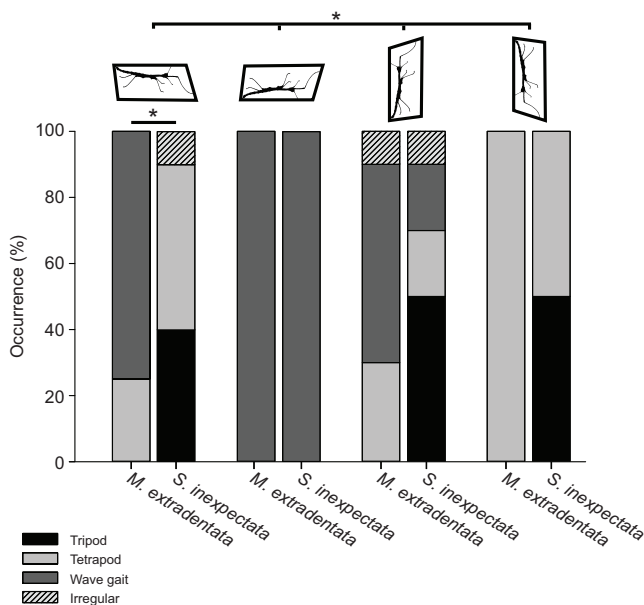


Fig. 3. Observations of walking gait patterns. Percentage of observations in which the two phasmid species, *M. extradentata* and *S. inexpectata* ($N=10$ for each species), applied different walking gait patterns in the four different posture situations. * $P < 0.05$; only significant comparisons are displayed.

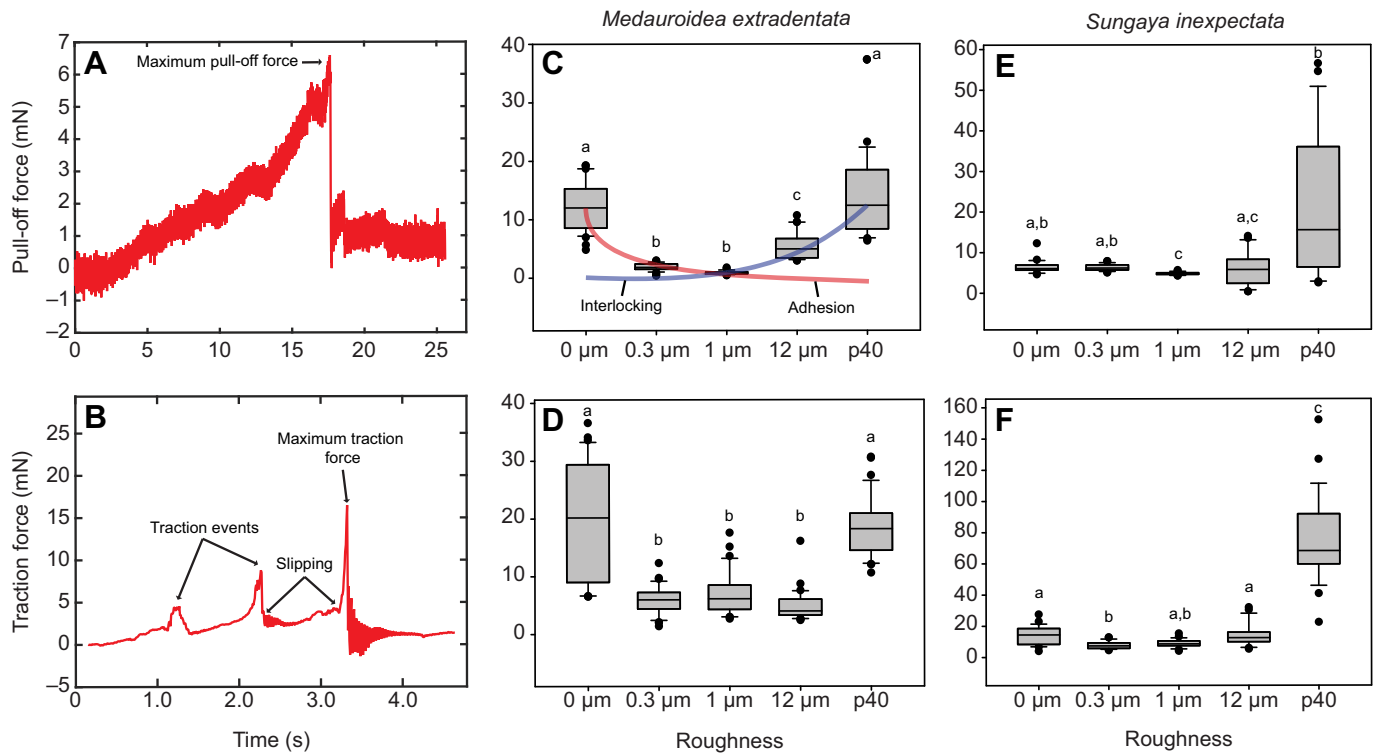


Fig. 4. Pull-off (top) and traction forces (bottom) for both species of stick insects on different substrate topographies. (A,B) Exemplary curves for measurements of pull-off (A) and traction (B) forces. (C,D) *Medauroidea extradentata* ($N=32$) pull-off force (C) and traction force (D). (E,F) *Sungaya inexpectata* ($N=25$) pull-off force (E) and traction force (F). Coloured lines indicate the hypothetical contribution of adhesion (red line) and mechanical interlocking (blue line), corresponding to the interpretation of the data. Roughness parameters for all substrates are provided in Salerno et al. (2017). Groups with the same letter are statistically equal. The boxes indicate 25th and 75th percentiles, the line within the boxes represents the median, and whiskers (error bars) define the 10th and 90th percentiles.

off forces on the 12 μm substrate were intermediate, but significantly different from those for all other substrates for this species (Kruskal–Wallis one-way ANOVA, $H=106.07$, d.f.=4, $N_{1,2}=32$, $P\leq 0.001$; Tukey's test, $P<0.05$).

Sungaya inexpectata revealed the highest pull-off forces on the roughest surface (15.5 ± 17.4 mN), but these were not significantly different from those for the 0 and 0.3 μm surfaces (Kruskal–Wallis one-way ANOVA, $H=37.773$, d.f.=4, $N_{1,2}=25$, $P\leq 0.001$; Tukey's test, $P>0.05$). On the smooth surface, pull-off forces were rather low (6.1 ± 1.5 mN) and only significantly different from those measured on the 1 μm surface (Kruskal–Wallis one-way ANOVA, $H=37.773$, d.f.=4, $N_{1,2}=25$, $P\leq 0.001$; Tukey's test, $P<0.05$). Comparison of traction forces between the 1 μm substrate and the 0 μm , 0.3 μm and p40 substrates, as well as between the p40 substrate and the 1 μm and 12 μm ones, showed that they were statistically different (Kruskal–Wallis one-way ANOVA, $H=37.773$, d.f.=4, $N_{1,2}=25$, $P\leq 0.001$; Tukey's test, $P<0.05$). In contrast to those in *M. extradentata*, pull-off forces in *S. inexpectata* on 1 μm (4.7 ± 0.3 mN) and 12 μm substrates (5.8 ± 4.2 mN) did not differ statistically (Kruskal–Wallis one-way ANOVA, $H=37.773$, d.f.=4, $N_{1,2}=25$, $P\leq 0.001$; Tukey's test, $P>0.05$).

Traction forces

Traction forces in *S. inexpectata* (Fig. 4F) were highest on the p40 substrate (68.2 ± 27.3 mN) and different from those on all other substrates (Kruskal–Wallis one-way ANOVA, $H=76.12$, d.f.=4, $N_{1,2}=25$, $P\leq 0.001$; Tukey's test, $P>0.05$). Besides this, the only statistical difference was found for the traction force (7.3 ± 2.3 mN), measured on 0.3 μm roughness; this was significantly lower than the

forces measured on 0 μm (14.5 ± 5.9 mN), 12 μm (12.5 ± 7.0 mN) and p40 substrates, but not the 1 μm one (8.5 ± 2.5 mN). Traction force on the 1 μm substrate showed differences only in comparison with that for the p40 substrate (Kruskal–Wallis one-way ANOVA, $H=76.12$, d.f.=4, $N_{1,2}=25$, $P\leq 0.001$; Tukey's test, $P<0.05$). Traction forces for *M. extradentata* were also significantly higher (Kruskal–Wallis one-way ANOVA, $H=99.54$, d.f.=4, $N_{1,2}=32$, $P\leq 0.001$; Tukey's test, $P>0.05$) for the smoothest and roughest surfaces (0 μm : 20.2 ± 10.1 mN; p40: 18.2 ± 5.0 mN), compared with those for all other surfaces (see Fig. 4D).

Influence of the claws on pull-off and traction forces

Pull-off and traction forces for all individuals with amputated claws are shown in Fig. 5. For the pull-off direction in *M. extradentata*, the forces measured for individuals with intact claws and for those with amputated claws were similar and showed no statistical difference, except on 0.3 μm and p40 roughness (Fig. 5A). For individuals with amputated claws, the pull-off forces were significantly lower than for those with claws on the 0.3 μm substrate (1.3 ± 0.5 mN; Mann–Whitney rank sum test, $U=285.00$, $T=1267.00$, $N_{1,2}=32$, $P=0.002$) and on the p40 substrate (0.7 ± 0.3 mN; Mann–Whitney rank sum test, $U=0.00$, $T=1552.00$, $N_{1,2}=32$, $P\leq 0.001$). Comparison of the pull-off forces (Fig. 5B) in *S. inexpectata* only revealed significantly lower values for individuals without claws on the p40 substrate (6.2 ± 3.2 mN; Mann–Whitney rank sum test, $U=143.00$, $T=807.00$, $N_{1,2}=25$, $P\leq 0.001$). Traction force comparisons for both species (Fig. 5C,D) revealed no statistical differences on the three least rough surfaces (0 μm , 0.3 μm and 1 μm), but showed significantly higher forces on the 12 μm surface (*M. extradentata*: 6.0 ± 1.2 mN,

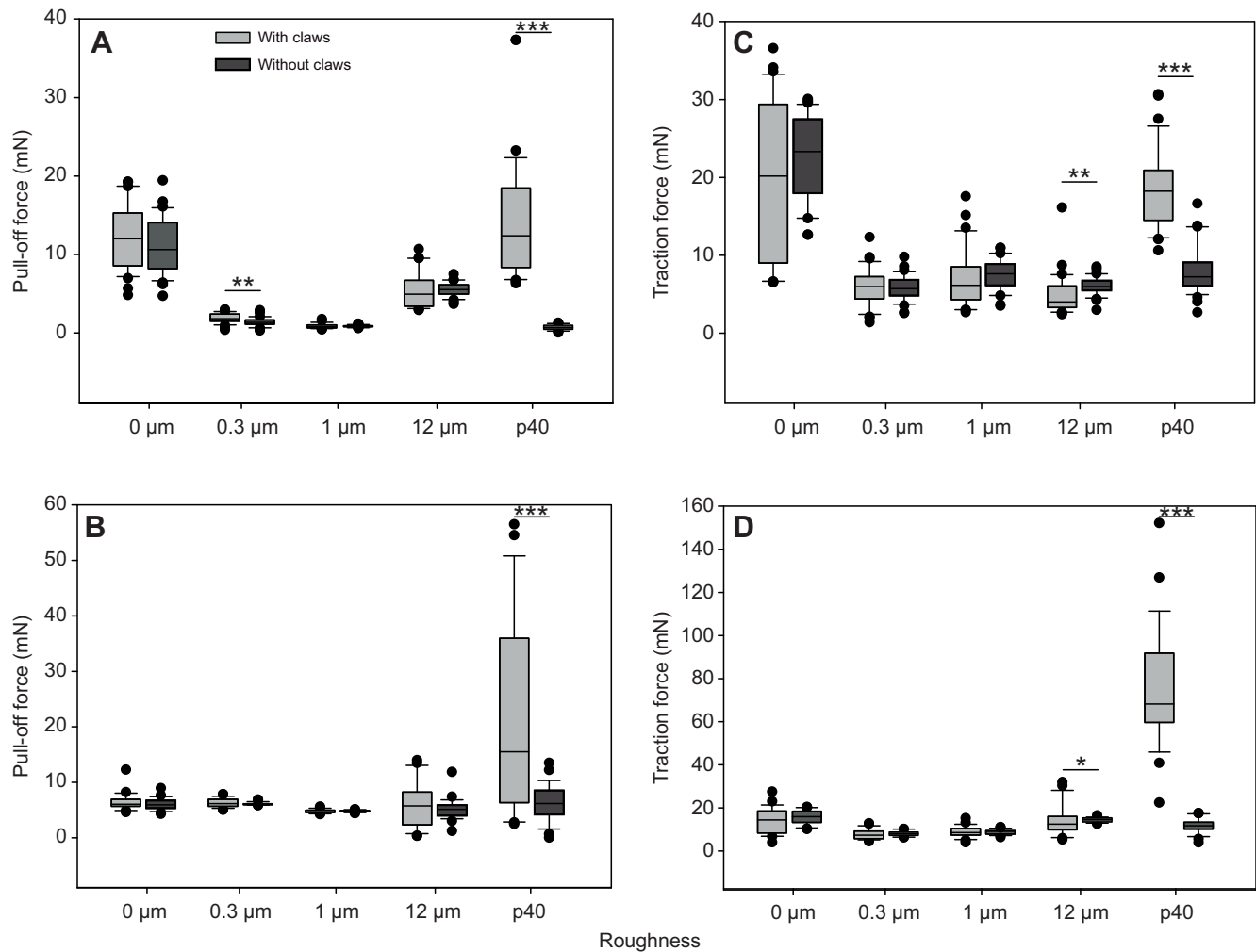


Fig. 5. Pull-off (left) and traction forces (right) for both species of stick insects, with intact claws and with amputated claws, on different substrate topographies. (A,C) *Medauroidea extradentata* ($N=32$ for each group with and without claws) pull-off force (A) and traction force (C). (B,D) *Sungaya inexpectata* ($N=25$ for each group with and without claws) pull-off force (B) and traction force (D). Roughness parameters for all substrates are provided in Salerno et al. (2017). * $P<0.05$, ** $P<0.01$, *** $P<0.001$; only significant comparisons are displayed. The boxes indicate 25th and 75th percentiles, the line within the boxes represents the median, and whiskers (error bars) define the 10th and 90th percentiles.

Mann–Whitney rank sum test, $U=300.00$, $T=828.00$, $N_{1,2}=32$, $P=0.005$; *S. inexpectata*: 14.6 ± 1.0 mN, Mann–Whitney rank sum test, $U=206.00$, $T=531.00$, $N_{1,2}=25$, $P=0.04$) and significantly lower forces for individuals without claws on the p40 substrate (*M. extradentata*: 7.2 ± 3.0 mN, Mann–Whitney rank sum test, $U=30.00$, $T=1522.00$, $N_{1,2}=32$, $P\leq 0.001$; *S. inexpectata*: 11.7 ± 3.3 mN, Mann–Whitney rank sum test, $U=0.00$, $T=950.00$, $N_{1,2}=25$, $P\leq 0.001$).

Differences between species

Comparison of the traction and pull-off forces between the two species (Fig. 6) revealed highly significant differences in the pull-off forces on substrates with $0\ \mu\text{m}$, $0.3\ \mu\text{m}$ and $1\ \mu\text{m}$ roughness. On the smooth substrate, the pull-off forces were higher for *M. extradentata* (Mann–Whitney rank sum test, $U=65.00$, $T=390.00$, $N_{S.inexpectata}=25$, $N_{M.extradentata}=32$, $P\leq 0.001$), but *S. inexpectata* had higher pull-off forces on the $0.3\ \mu\text{m}$ (Mann–Whitney rank sum test, $U=0.00$, $T=1125.00$, $N_{S.inexpectata}=25$, $N_{M.extradentata}=32$, $P\leq 0.001$) and $1\ \mu\text{m}$ (Mann–Whitney rank sum test, $U=0.00$, $T=1125.00$, $N_{S.inexpectata}=25$, $N_{M.extradentata}=32$, $P\leq 0.001$) substrates. *Sungaya inexpectata* showed considerably higher pull-off forces on the $0.3\ \mu\text{m}$ and $1\ \mu\text{m}$ substrates

(median more than 3 times higher on $0.3\ \mu\text{m}$ and more than 4 times higher on $1\ \mu\text{m}$) in comparison to *M. extradentata*. Traction forces on the smooth substrate showed no significant difference between the two species (Mann–Whitney rank sum test, $U=281.00$, $T=606.00$, $N_{S.inexpectata}=25$, $N_{M.extradentata}=32$, $P=0.057$), but *S. inexpectata* had higher values on all other roughnesses. The forces produced by *S. inexpectata* were higher on the $0.3\ \mu\text{m}$ (Mann–Whitney rank sum test, $U=228.00$, $T=897.00$, $N_{S.inexpectata}=25$, $N_{M.extradentata}=32$, $P=0.006$) and $1\ \mu\text{m}$ substrates (Mann–Whitney rank sum test, $U=233.00$, $T=892.00$, $N_{S.inexpectata}=25$, $N_{M.extradentata}=32$, $P=0.007$). The other substrate comparisons revealed much higher values for *S. inexpectata*, with the median pull-off forces more than 3 times higher on the $12\ \mu\text{m}$ (Mann–Whitney rank sum test, $U=49.00$, $T=1076.00$, $N_{S.inexpectata}=25$, $N_{M.extradentata}=32$, $P\leq 0.001$) and p40 (Mann–Whitney rank sum test, $U=6.00$, $T=1119.00$, $N_{S.inexpectata}=25$, $N_{M.extradentata}=32$, $P\leq 0.001$) substrates.

DISCUSSION

Attachment system of Phasmatodea

Although the tarsal attachment system of stick insects has been the subject of some studies focusing on functional aspects of single

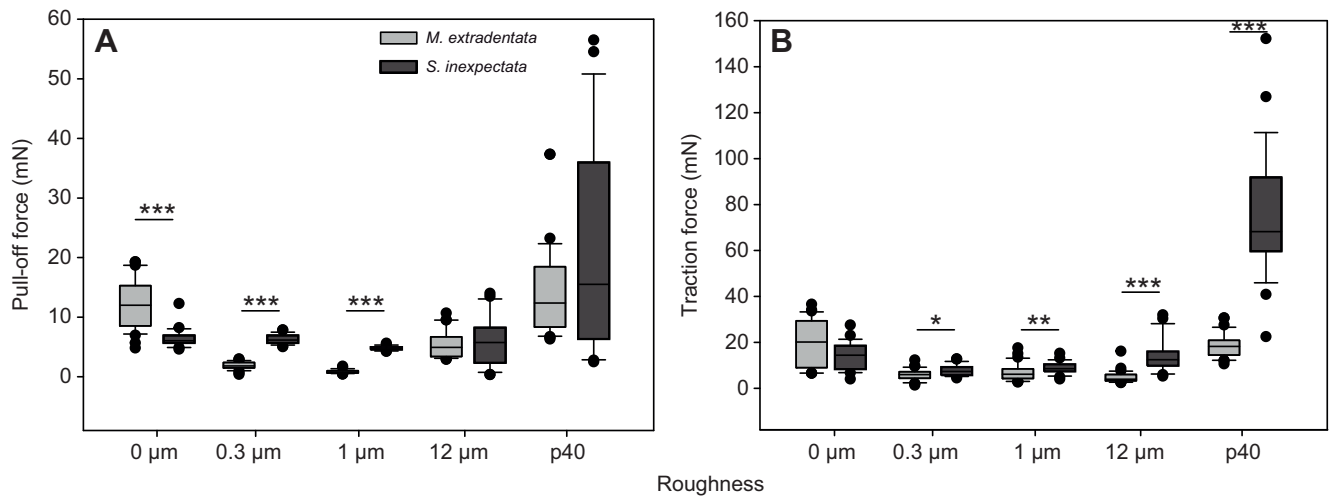


Fig. 6. Comparison of attachment forces of the two species of stick insects. (A) Pull-off forces (*S. inexpectata* $N=25$, *M. extradentata* $N=32$). (B) Traction forces (*S. inexpectata* $N=25$, *M. extradentata* $N=32$). Roughness parameters for all substrates are provided in Salerno et al. (2017). * $P<0.05$, ** $P<0.01$, *** $P\leq 0.001$; only significant comparisons are displayed. The boxes indicate 25th and 75th percentiles, the line within the boxes represents the median, and whiskers (error bars) define the 10th and 90th percentiles.

attachment pads (e.g. Bußhardt et al., 2012; Labonte and Federle, 2014; Labonte et al., 2014), the simultaneous contribution of the different components has not been investigated so far. We show for the first time how the different attachment structures are used in combination, under different behavioural situations and how the system performs as a whole. We connect the knowledge obtained from the attachment properties of single pads with the performance of the entire system.

In general, the results corroborate those for single pad measurements. The usage of arolia and euplantulae corresponds to the properties measured for the two attachment pad types (Labonte et al., 2014; Labonte and Federle, 2014) and the complementary function of the two different attachment pads and claws corresponds to previous results made on a biomimetic artificial model (Song et al., 2016). Differences between the functionality of nubby and smooth attachment structures also agree with previous single pad measurements (Bußhardt et al., 2012) in regard to the influence of surface roughness on attachment. However, the frictional anisotropy of the euplantulae (Bußhardt et al., 2012) does not explain their usage in some situations and might be connected with different scenarios, e.g. locomotion on twigs (see Gladun and Gorb, 2007), as discussed below.

The tarsus of Phasmatodea consists of five tarsomeres that bear four euplantulae on the proximal segments and an arolium and two claws on the pretarsus (e.g. Beutel and Gorb, 2001, 2006, 2008; Büscher et al., 2018a,b). While the arolia of *Timema*, a lineage that split very early in the evolution of Phasmatodea and represents the sister group to the remaining Euphasmatodea (Bradler, 2009; Bradler et al., 2014, 2015; Büscher et al., 2018a), are covered by short acanthae, the euphasmatodean arolia are entirely smooth (Beutel and Gorb, 2006, 2008). The species studied here have the usual setup of attachment devices and represent the two most common microstructures in Euphasmatodea: nubby and smooth (Fig. 1).

Attachment pad usage

Our first aim was to examine the usage of the different parts of the tarsal attachment system. The attachment system of the tarsi always involves the arolium in both species studied (Fig. 1, 2). Depending on the posture, different numbers of euplantulae can be additionally brought into contact, but always in a sequence from distal to proximal. Stick insects in a resting position are reported to use the

proximal euplantulae (Labonte and Federle, 2014). However, we observed that walking stick insects always use distal euplantulae (two for *S. inexpectata* and sometimes three for *M. extradentata*) and the arolia (Fig. 2A,E). When resting on even ground, the legs primarily provide body weight support, and the attachment system acts as an anchor to the ground. However, when walking, the legs and attachment system also facilitate propulsion to push the body forward, facilitating body weight support and medio-lateral balance (Dallmann et al., 2016). This involves forces acting in different directions at the same moment for the different leg pairs (Dallmann et al., 2016); however, the number of euplantulae observed in contact was constant in all three leg pairs. Therefore, we conclude that each tarsus experiences shear and normal forces simultaneously, but to a different extent. As the same number of attachment pads is used in upright walking in every leg, the combined use of the arolium and euplantulae provides the right amount of attachment to the ground for propulsion generation, whilst enabling fast detachment to facilitate fast stepping.

In situations where attachment pads act against gravity, especially when hanging from the ceiling, the load-sensitive euplantulae (Gorb et al., 2000; Gorb and Scherge, 2000; Labonte et al., 2014; Labonte and Federle, 2014) are not used. While hanging from the ceiling, the tarsus is stretched. Additionally, the euplantulae need to be brought into contact with the substrate and load needs to be applied against gravity. Even in *M. extradentata*, a species with smooth euplantulae, none of the euplantulae was used while hanging upside down, although Bußhardt et al. (2012) reported essentially higher adhesion for the smooth euplantulae of this species in comparison to that with nubby euplantulae. Likewise, in vertical climbing, the euplantulae were similarly not, or rarely, used on legs that were in contact above the centre of mass (forelegs for walking upwards, hindlegs for walking downwards). The stretching of the tarsal chain probably prevents the animal from bringing the euplantulae into contact and applying load on the euplantulae, even though frictional coefficients measured on single euplantulae (Bußhardt et al., 2012) are larger for pulls than for pushes. As both species use euplantulae for pushing on flat surfaces and the traction forces would be higher in the pulling direction, higher friction coefficients in pulling can be beneficial for attachment on curved substrates in which the tarsus is able to clasp around the substrate (see Voigt et al., 2017). Apparently, on a flat

vertical surface, legs above the centre of mass primarily provide adhesion to counterbalance torque, thus enabling proper attachment of the whole animal (Goldman et al., 2006; Clemente and Federle, 2008; Eberhard et al., 2009; Labonte and Federle, 2014). In contrast, the legs situated below the centre of mass generate traction and therefore utilise all euplantulae (Clemente and Federle, 2008; Labonte and Federle, 2014). These legs also exploit the load produced by the torque from the animal's centre of mass. A similar differentiation between the usage of mainly arolia in pulling legs and mainly euplantulae in pushing legs has been shown for cockroaches on vertical surfaces (e.g. Clemente and Federle, 2008; Clemente et al., 2009). The minor differences in the frequency of euplantula contacts observed in the two examined species might be related to the body shape of the animals. While the body of *M. extradentata* is typically elongated, that of *S. inexpectata* is shorter and more robust. Consequently, the location of the centre of mass differs in the two species: in *M. extradentata*, it is located between the hindleg coxae (Theunissen et al., 2015, based on linear measurements) and can be assumed in *S. inexpectata* to be more anterior, based on the centre of mass of *Aretaon asperrimus*, which has a similar body shape (Theunissen et al., 2015).

The coordination of the legs in stick insects during walking is well studied (e.g. Wilson, 1966; Graham, 1985; Cruse, 1990; Bässler and Büschges, 1998; Grabowska et al., 2012; Theunissen et al., 2015) in terms of sensorics, neuronal control, muscle activity and behavioural adaptability. To date, attachment has not been compared with walking pattern analyses, even though attachment at different inclines or posture situations is of importance. Our results clearly indicate that the occurrence of walking gait patterns in horizontal upright walking is species specific. *Medauroidea extradentata* applies fewer legs in horizontal walking simultaneously compared with *S. inexpectata*, which often uses tripod gait. This difference probably arises from the reduced antennae in *M. extradentata* (Mujagic et al., 2007), rather than from the difference in the AMS between the species. Usually, antennae in stick insects are long and serve tactile sensing of the near-range environment (Dürr et al., 2001; Dürr and Bläsing, 2001; Schütz and Dürr, 2011; Krause and Dürr, 2012). In contrast, *M. extradentata* often use the elongated front legs for sensing the environment (e.g. Karg et al., 1991; Berg et al., 2013; Theunissen et al., 2015) and therefore moves fewer legs simultaneously. According to our statistical analysis, all other differences are not related to the species. But, differences on the vertical surfaces might still be influenced by the AMS, which is specific for the two species. Generally, the gaits applied by *S. inexpectata* show a higher percentage of tripod patterns on the vertical plane (in both directions) than on the horizontal plane. For nubby AMS, the high friction, resulting from the gravitational force, might enhance attachment of individual tarsi and seems to be beneficial in this posture situation (Labonte et al., 2014; Grohmann et al., 2015). On the ceiling, the tarsi experience no load caused by the body weight. Rather, high tensile forces acting on the tarsi result in the use of arolia, not euplantulae (Labonte et al., 2014). Although the arolia are well adapted for use in this situation, the insect's weight needs to be compensated for by the simultaneous use of the tarsi of five legs at the same time. While phasmids move only one leg at a time on the ceiling (similar to some bugs: Gorb and Heepe, 2017), some flies move two legs (Gorb, 2001, 2005; Niederegger et al., 2002; Gorb and Heepe, 2017) and some beetles even use tripod gait on the ceiling (Gorb and Heepe, 2017). Consequently, the number of legs in contact with the ceiling depends on the safety factor (attachment force divided by body weight), which reflects the relationship between the adhesive performance of an animal and its body weight. According to

experiments with insects representing different safety factors (Gorb and Heepe, 2017), species with safety factors higher than 10 are able to use three legs at once in the swing phase, i.e. employ tripod gait on the ceiling. Species with safety factors from 4 to 5 are able to detach two legs simultaneously, and those with safety factors below 3 attach five legs to the ceiling and only use one leg at a time in swing phase. Obviously, the dynamic adhesion of both species necessitates many attachment sites. The safety factors of both species studied here are seemingly below 3 when walking on the ceiling, as only one leg was moved during locomotion (Gorb and Heepe, 2017). The fifth euplantula, which is only present in *S. inexpectata*, was not used in any of the situations examined herein. This attachment pad possibly contributes to attachment in situations different from walking on even substrates, e.g. walking on curved substrates or on thin stems (Gladun and Gorb, 2007).

Attachment system performance

Our second aim was to investigate the performance of the entire attachment system on substrates with different levels of roughness. Pull-off and traction forces generally depend on the roughness of the substrate that the attachment pads face (e.g. Gorb, 2001; Arzt et al., 2003; Gorb et al., 2005; Bullock and Federle, 2011; Scholz et al., 2010; Wolff and Gorb, 2012b). The majority of the insect species studied show higher forces on smooth substrate than on surfaces with fine roughness (0.3–1.0 μm), independent of the direction of measurement. Because of the rather soft properties of attachment pads, they are able to generate a comparably high contact area on the smooth surface (Perez-Goodwyn et al., 2006; Peisker et al., 2013; Bennemann et al., 2014). The surface irregularities of the slightly rough substrates probably prevent optimal contact formation, as the cuticle does not adapt sufficiently to the substrate profile (Arzt et al., 2003; Persson and Gorb, 2003).

Pull-off forces

In our pull-off measurements, only arolia were in touch with the substrate (see Movie 2) and so we obtained pull-off forces that reflect the adhesive properties of only this attachment pad. Nevertheless, *M. extradentata* showed the best attachment performance on both the smooth and most corrugated substrates. On smooth surfaces, the arolia are able to generate attachment, but the claws cannot interlock (Song et al., 2016). In contrast, on the roughest substrate, the claws easily interlock. However, the soft attachment pads generate comparably low contact area with the rougher substrate (Jiao et al., 2000). On surfaces with minor roughness, i.e. 0.3 and 1.0 μm , the claws cannot interlock, as the claw tip diameter exceeds the gaps in the surface texture (Song et al., 2016; Patrick et al., 2018). The attachment pads are probably unable to deform without gaps and therefore do not generate a comparably high contact area (Arzt et al., 2003; Persson and Gorb, 2003). However, pull-off forces on the 12 μm roughness were intermediate between those obtained on the other roughnesses. Potentially, the claw tip could be able to interlock with the surface irregularities at this level of roughness, but the individuals with amputated claws still revealed similar or even higher (Fig. 5) attachment forces. As there was no difference between the attachment forces of specimens with and without claws, only between the different levels of roughness within the same groups, the attachment pads possibly generate more actual contact area on the substrate with 12 μm roughness in comparison to 0.3 μm and 1 μm roughness. The higher attachment force on 12 μm roughness potentially indicates a higher actual contact area (Arzt et al., 2003; Persson and Gorb, 2003). Interestingly, the performance of the claws on p40 roughness in *M. extradentata* is similar to the attachment

performance of arolia on the smooth substrate. The complementary function of claws and attachment pads provides proper attachment to both smooth and very rough surfaces (Song et al., 2016). For *M. extradentata*, pull-off forces reveal very high safety factors (median of attachment force per median of body mass) on the smooth (safety factor approximately 22.6) and rough substrate (approximately 23.3). *Sungaya inexpectata*, with its reduced arolia (Büscher et al., 2019) compared with that of *M. extradentata*, showed lower adhesion on the smooth surface. Adhesive secretions, present in phasmids (Drechsler and Federle, 2006; Dirks et al., 2010; Dirks and Federle, 2011), may additionally mediate attachment on rough surfaces. This can contribute to the performance of the attachment pads by levelling roughness caused by small irregularities of the profile (e.g. Dixon et al., 1990; Gorb, 2001; Kovalev et al., 2013). However, in general, both species across all experiments showed lower performance on coarse than on fine rough surfaces. As the projected pad area of the arolium (Büscher et al., 2019), and therefore probably also its actual contact area, is smaller in *S. inexpectata*, pull-off forces on the smooth substrate are lower in comparison with those for *M. extradentata* (Fig. 6A). This, however, does not explain the significantly higher pull-off forces of *S. inexpectata* in comparison with those for *M. extradentata* on the 0.3 μm and 1 μm rough substrates. Possible reasons for this are related to different properties of adhesive fluids in both species that should affect these small roughnesses (Drechsler and Federle, 2006). Other differences in material properties of the arolia, e.g. Young's modulus, might cause similar effects, as has been previously shown for orthopterans (Perez-Goodwyn et al., 2006). Stiffer materials are less flexible and therefore do not adapt to roughness as well (Peisker et al., 2013).

Traction forces

Our third aim was to compare the attachment performance of species employing smooth and nubby AMS. The euplantulae, which are the only attachment pads showing such a morphological difference between species, were used only in the traction direction. The general trends of attachment forces are similar to those for the pull-off direction, e.g. highest attachment force in *M. extradentata* on the smoothest and most corrugated substrate. In the shear direction, the claws performed well on the 12 μm substrate, as has been previously shown for the beetle species *Pachnoda marginata* (Bußhardt et al., 2014) and *Gastrophysa viridula* (Bullock and Federle, 2011). The major differences between the two species are visible with increasing degree of roughness (Fig. 6B), with the biggest difference for p40, possibly explained by the contribution of the claws. The performance of the claws depends on the radius of the claw tip curvature as well as on the curvature of the substrate irregularities (e.g. Dai et al., 2002; Song et al., 2016; Patrick et al., 2018). The claws of *M. extradentata* (claw tip diameter $d_t=7.16\pm 1.77 \mu\text{m}$) are significantly sharper (t -test, $t=-7.36$, d.f.=18, $N_{1,2}=10$, $P\leq 0.001$) than those of *S. inexpectata* ($d_t=14.06\pm 2.76 \mu\text{m}$) (see Fig. S1), but the attachment forces are higher in *S. inexpectata*. It is likely that the claws of both species equally facilitate proper interlocking with the substrate. Accordingly, the higher traction forces in *S. inexpectata* are caused by other factors, e.g. differences in the musculature. The differences of traction force on surfaces with intermediate levels of roughness possibly arise from the presence of nubs on the euplantulae of *S. inexpectata*, which are adapted to a broad range of surface roughnesses (Bußhardt et al., 2012). The traction forces on 1 μm and 12 μm substrates were larger in *S. inexpectata*. The nubby euplantulae of this species possess cuticular protuberances that can interlock with the surface profile of some rough substrates (Bußhardt et al., 2012; Labonte and Federle, 2014; Labonte et al., 2014) and generate

friction mediated by interlocking. In contrast, the euplantulae of *M. extradentata* are adapted to smooth surfaces and probably do not generate sufficient contact area on rough surface profiles (Bußhardt et al., 2012).

Secondary effects of the claws in attachment

Obviously, mechanical interlocking of phasmatodean attachment devices with the substrate plays a major role for substrates with a certain minimum roughness. On smooth surfaces, the claws do not interlock and the attachment pads (arolia and euplantulae) are the only mechanism providing grip on the surface (e.g. Gorb, 2001). Detachment and attachment of bladder-like pads can potentially be controlled by haemolymph pressure (Denning et al., 2014). The arolium of stick insects contains a large haemolymph-filled region that possibly contributes to this control (Bennemann et al., 2011, 2014). As the claws lie on the dorsal side of the arolium they could potentially apply additional pressure on the arolium dorsally. However, judging from the acquired force data for individuals with and without claws, there is no secondary effect of the claws on the performance of the arolium. The major differences in pull-off and traction forces between animals with and without claws appear mainly on very rough surfaces (p40; Fig. 5) and are obviously connected with the mechanical interlocking of the claws themselves.

Conclusions

In this study, we investigated the usage of the attachment system consisting of claws and adhesive/frictional pads of two species of stick insects and examined the attachment forces in the pull-off and traction direction on substrates of different roughness. We aimed to understand the interplay between the different parts of the system and the functional influence of two different types of euplantular attachment microstructures on the overall attachment performance. From the obtained data we draw the following conclusions. (i) The distal arolia are used in every situation, but the proximal euplantulae are employed primarily in situations where the body weight can generate load on them (upright walking, vertical climbing). Although previous studies show larger friction coefficients for pulls than for pushes, the conformation of the tarsal apparatus prevents the euplantulae from getting in contact and load being applied on them if the tarsus is straight and stretched. (ii) Arolia and euplantulae provide attachment on smooth surfaces, while claws provide mechanical interlocking at surface roughnesses over 12 μm . The overall attachment forces in both the traction and pull-off direction are large at very low and very high surface roughness, but are lowest in the intermediate range of substrate roughness (0.3–1.0 μm). (iii) The species with smooth euplantulae (*M. extradentata*) attaches better to smooth surfaces in comparison to the species with nubby euplantulae (*S. inexpectata*). The species with nubby euplantulae performs better on micro-rough surfaces. This agrees with previous data on single euplantulae.

Acknowledgements

We thank Dr Lars Heepe, Dr Sebastian Büsse, Dennis Petersen and Chuchu Li (Department of Functional Morphology and Biomechanics, Kiel University, Germany) for their technical help and fruitful discussions. Dr Sven Bradler (Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology, University of Göttingen, Germany) and Daniel Dittmar (Berlin, Germany) are thanked for providing specimens for the original laboratory cultures of the species used. We also acknowledge Braxton Jones (Department of Biological Sciences, Macquarie University, Australia) for proofreading the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.H.B., S.N.G.; Methodology: T.H.B., S.N.G.; Validation: T.H.B., S.N.G.; Formal analysis: T.H.B.; Investigation: T.H.B.; Resources: T.H.B., S.N.G.; Data curation: T.H.B., S.N.G.; Writing - original draft: T.H.B.; Writing - review & editing: T.H.B., S.N.G.; Visualization: T.H.B.; Supervision: S.N.G.; Project administration: T.H.B., S.N.G.; Funding acquisition: S.N.G.

Funding

This work was funded by the German Science Foundation (Deutsche Forschungsgemeinschaft grant GO 995/34-1).

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.209833.supplemental>

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