

RESEARCH ARTICLE

Evidence for a sexually selected function of the attachment system in bedbugs *Cimex lectularius* (Heteroptera, Cimicidae)

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ABSTRACT

Attachment to surfaces is a major aspect of an animal's interaction with the environment. Consequently, shaping of the attachment system in relation to weight load and substrate is considered to have occurred mainly by natural selection. However, sexual selection may also be important because many animals attach to their partner during mating. The two hypotheses generate opposing predictions in species where males are smaller than females. Natural selection predicts that attachment ability will scale positively with load, and hence body size, and so will be larger in females than males. Sexual selection predicts attachment forces in males will be larger than those in females, despite the males' smaller size because males benefit from uninterrupted copulation by stronger attachment to the female. We tested these predictions in the common bedbug *Cimex lectularius*, a species in which both sexes, as well as nymphs, regularly carry large loads: blood meals of up to 3 times their body weight. By measuring attachment forces to smooth surfaces and analysing *in situ* fixed copulating pairs and the morphology of attachment devices, we show that: (i) males generate twice the attachment force of females, despite weighing 15% less; (ii) males adhere to females during copulation using hairy tibial adhesive pads; (iii) there are more setae, and more setae per unit area, in the pads of males than in those of females but there is no difference in the shape of the tarsal setae; and (iv) there is an absence of hairy tibial attachment pads and a low attachment force in nymphs. These results are consistent with a sexually selected function of attachment in bedbugs. Controlling sperm transfer and mate guarding by attaching to females during copulation may also shape the evolution of male attachment structures in other species. More generally, we hypothesise the existence of an arms race in terms of male attachment structures and female counterparts to impede attachment, which may result in a similar evolutionary diversification to male genitalia.

KEY WORDS: Adhesive setae, Centrifugal force test, Fossula spongiosa, Resilin, Safety factor, Sexual conflict, Tibial setae

INTRODUCTION

Attachment to surfaces is a vital component of an animal's interaction with its natural environment. Consequently, attachment structures and the ability to adhere to plant, host, ground or artificial surfaces has

been a vibrant research area for the last few decades in reptiles and amphibians (e.g. Autumn et al., 2006; Tian et al., 2006), but particularly in insects (e.g. Stork, 1980a; Gorb, 1998, 2001; Federle et al., 2002; Betz, 2002; Gorb and Gorb, 2002, 2009; Voigt et al., 2007, 2017a; Prüm et al., 2011, 2013; Gorb et al., 2014; Yanoviak et al., 2016).

In insects, connection to the partner during mating had been recognised as another function of attachment. For example, attachment structures were limited to, or more elaborated in males, or were hypothesised or shown to facilitate uninterrupted copulation by clasping, such as in leaf beetles, ladybirds, diving beetles and mirid bugs (e.g. Plateau, 1872; Schanz, 1953; Stork, 1980a,b, 1981; Pelletier and Smilowitz, 1987; Aiken and Khan, 1992; Gorb, 2001; Bergsten et al., 2001; Voigt et al., 2008, 2017b, 2019; Bullock and Federle, 2009; Karlsson Green et al., 2013; Heepe et al., 2017).


The question of whether natural or sexual selection primarily shapes the attachment system, or which aspects of it, seems fundamental to our understanding of the origin and function of attachment systems. This question can be addressed experimentally because the two concepts make opposing predictions. Attachment forces that primarily function in naturally selected contexts should scale positively with body size (e.g. Gorb, 2001; Varenberg et al., 2010; Labonte et al., 2016) because the load carried by the animal on surfaces (body weight) was the primary selective agent to optimise attachment devices. Therefore, sexual differences in attachment should be explained mainly by sexual differences in body size, and are probably additionally influenced by selection having acted on females because they carry the load of the developing eggs and of the copulating male (see Watson et al., 1998, for an example of the cost of the latter). In contrast, if sexual selection has acted on male attachment during copulation by maintaining sperm transfer despite dislodgement attempts by rival males or by avoidance movements of females or if males were better able to mate-guard, we would expect larger attachment forces in males than in females, regardless of their size differences.

Here, we tested these competing predictions in the common bedbug *Cimex lectularius* L. (Heteroptera, Cimicidae), a species where males are smaller than females. Both sexes, as well as nymphs, take blood meals in the wild regularly, up to one blood meal every 3 days in adults (Reinhardt et al., 2010). Blood meals generate loads of 3–5 times the individual's body mass, generated within approximately 15 min (the duration required to obtain a full blood meal). Female *C. lectularius*, on average, take 3 times larger blood meals than males (Titschack, 1930). Last (fifth) instar nymphs also take larger blood meals than males (Titschack, 1930). A naturally selected function of attachment structures, therefore, predicts the following rank order of attachment forces: females>nymphs>males. Sexual selection predicts an attachment rank order of males>females>(not sexually active) nymphs.

The attachment structure on the tibia of bedbugs in the Cimicomorpha (Heteroptera), known as fossula spongiosa

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(Weirauch, 2007), consists of tenent setae. It is a representative of the 'hairy adhesive pads' (Gorb, 2001) and is here broadly defined as a tibial attachment pad (TAP). Its presence in the Cimicidae has previously been mentioned (Wigglesworth, 1938; Lee, 1955; Walpole, 1987) and described (Kim et al., 2017; Baker and Goddard, 2018). Kim et al. (2017) reported that adult male *C. lectularius* possessed more setae in the foreleg and midleg TAP than adult females and that hairy TAPs are absent in nymphs. Because a naturally selected function of attachment would be inconsistent with such a reported absence of TAPs in nymphs and with their stronger elaboration in males, we here took a more direct approach and directly measured attachment forces generated by TAPs in living animals. We also examined four additional aspects relevant to biological variation in attachment force. As male attachment during copulation would largely concern attachment to the hydrophobic female cuticle, (i) we tested whether males have a stronger attachment on hydrophobic than on hydrophilic surfaces, compared with females. (ii) We observed the position of TAPs in males and females during copulation (a sexually selected context) and compared it with the position during blood sucking (a naturally selected context). (iii) We assessed which sexual differences in TAP morphology correlate with sexual differences in attachment forces and, related to that, (iv) we tested whether nymphs, which do not have TAPs, indeed have lower attachment forces. The dimorphism of attachment forces and the other four aspects of the attachment were all consistent with a sexually selected function of TAP attachment in bedbugs.

MATERIALS AND METHODS

Study animals

Bedbugs originated from a large, outbred laboratory population (called S1) of more than 1000 individuals. Originally collected in London, UK, the colony had been in culture in a laboratory at the University of Sheffield, UK, for more than 5 years at the time of the study, and before that for ca. 40 years in a laboratory in London. In our study, bedbugs were mass reared in vials in incubators at 25°C and 70% relative humidity (for details, see Reinhardt et al., 2003). In this colony, females were 4.9% larger than males (mean±s.d. pronotum width: females 1.54±0.07 mm, males 1.46±0.07 mm; $n=30$ each).

Centrifugal force measurements

We define 'attachment' as a non-specific term (likely to comprise friction, adhesion and any behavioural–physiological effects). To study the attachment ability of bedbugs, we took fully sclerotised adults and 5th stage nymphs from the colonies. Tested individuals had not fed for at least 10 days, judging from experience based on their body size and shape (Wintle and Reinhardt, 2008) and gut filling. All animals were intact and had all six legs.

To assess bedbug attachment forces generated on epoxy resin, normal (hydrophilic) glass and silanised (hydrophobic) glass discs, a computer-controlled centrifugal force tester was used (Tetra Zentri-01-P, Tetra GmbH, Ilmenau, Germany) (see Gorb et al., 2001, for a description of the procedure). Prior to experiments, the glass was cleaned, hydrophobised (silanised) and characterised according to Voigt and Gorb (2010). The epoxy resin disc was prepared by two-step moulding of a clean glass surface (Spurr, 1969; Gorb, 2007). The physico-chemical properties of the test substrates are summarised in Table 1.

Prior to the centrifugal experiment, each bedbug was weighed using an analytical balance (AG 204 Delta Range, Mettler Toledo GmbH, Greifensee, Switzerland). Individual bedbugs were placed

Table 1. Wettability and free surface energy of test substrates used to measure attachment forces of bedbugs, *Cimex lectularius*

Substrate	Free surface energy	Polar component	Disperse component	CA (deg)
Normal glass	52.0 mN mm ⁻¹	34.6 mN mm ⁻¹	17.4 mN mm ⁻¹	42
Silanised glass	11.4 mN mm ⁻¹	1.8 mN mm ⁻¹	9.6 mN mm ⁻¹	108
Spurr resin	27.2 mN mm ⁻¹	4.0 mN mm ⁻¹	23.2 mN mm ⁻¹	91.3

The contact angle measuring device OCAH200 and SCA20 3.7.4 software (Data-Physics Instruments GmbH, Filderstadt, Germany) were used to estimate the wettability of test substrates by contact angle (CA) measurements with Aqua Millipore water. Free surface energy and its polar and disperse component were calculated according to Voigt and Gorb (2010).

on the horizontal surface of the centrifuge drum, which was covered with the epoxy resin or glass substrate, and continuously accelerated from 50 to 3000 rpm (0.14–540 g) in 20 s. Laboratory conditions were 23.7±1.7°C temperature and 47.3±10.0% relative humidity (mean±range). To examine sexual and ontogenetic differences on standard smooth surfaces, we tested eight males, eight females and four 5th instar nymphs on epoxy resin 10 times each. To assess whether the substrate wettability affected the attachment difference between males and females, we tested five males and five females 10 times each on hydrophilic and hydrophobic glass (Table 1). Given the substantial forces or pressures necessary to scratch the surface of glass (>1000 MPa) or Spurr resin (100 MPa), scratching by claws and their subsequent interlocking in the scratches is negligible; attachment will largely, if not exclusively, be determined by the action of the adhesive pads (TAPs).

Use of TAPs during feeding and mating

To observe the position of the TAP of bedbugs during copulation, we placed two pairs on filter paper and allowed them to mate. Approximately 20 s after mating started, the filter paper with the copulating pair was fixed in liquid nitrogen and further observed with cryo-scanning electron microscopy (see 'Cryo-SEM', below). In order to account for the possibility that the fixation procedure would change the position of the male TAP on the female, we also observed individuals directly. We randomly selected six males and six females from the colony and observed them under a stereoscope (Leica EZ4, Leica Microsystems Schweiz AG, Heerbrugg, Switzerland) at 8.5–35× magnification. We placed them individually on the forearm of a human volunteer (K.R., D.V.). For walking and feeding, we recorded for each leg whether TAPs and claws were in contact with bare skin, skin folds and hairs. We then placed the six males and six females in a Petri dish lined with filter paper and allowed them to mate. We recorded whether TAPs and claws were in contact with the female abdominal surface, separately for each leg.

Light microscopy

Fresh tarsi of adult bedbugs were cut off with a razor blade, mounted on glass slides in polyvinylalcohol (Moviol) and covered with glass cover slips. Samples were observed under a fluorescence microscope (Zeiss Axioplan) equipped with an HBO 103 mercury vapour lamp and XBO 75 xenon shortarc lamp (Carl Zeiss MicroImaging GmbH, Jena, Germany) and an integrated digital video-camera (AxioCam MRc, AxioVision GmbH, München-Hallbergmoos, Germany). Fluorescence microscopy was employed to reveal the possible existence of the elastic protein resilin in the pretarsal structures. A non-exclusive characteristic of this protein is its auto-fluorescence emission maximum at 420 nm (Andersen and

Weis-Fogh, 1964) when excited with UV light. Digital images obtained at green (excitation 512–546 nm, emission 600–640 nm), red (excitation 710–775 nm, emission 810–890 nm) and UV wavelengths (excitation 340–380 nm, emission 425 nm) were superimposed (Gorb, 1999).

Cryo-SEM

Cryo-scanning electron microscopes (Hitachi S-4800, Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a Gatan ALTO 2500 cryo-preparation system (Gatan Inc., Abingdon, UK); and Zeiss SUPRA 40VP-31-79 (Carl Zeiss SMT Ltd, Oberkochen, Germany) equipped with an EMITECH K250X cryo-preparation unit (Quorum Technologies Ltd, Ashford, Kent, UK) were used to observe the attachment structures. Fresh samples of bedbug legs cut using a razor blade and of mating couples shock-frozen in liquid nitrogen were mounted on metal holders using Tissue-Tek O.C.T.TM Compound (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands), frozen in the cryo-preparation chamber at -140°C , sublimated for 3 min at -90°C (for 25 min at -70°C in the case of mating couples) to remove contamination by condensed ice crystals, sputter-coated with gold–palladium or platinum (about 6 nm thickness) and examined in a frozen state in the cryo-SEM at 3–5 kV and -100 to -120°C . Obtained images were stored for later inspection of the attachment structures (see ‘Parameters examined and statistical analysis’, below).

To visualise footprints on the smooth substrate after detachment, live female bedbugs were held by tweezers and their feet were allowed to make contact with gold–palladium-coated resin replicas of glass. These footprints were examined as described above (for a detailed description of the method, see Gorb, 2006, and Gorb et al., 2012).

Parameters examined and statistical analysis

Cryo-SEM micrographs were used to characterise the attachment structures of those animals that were used in the centrifugal force tests. We measured the maximum length and width of the TAP and setae, separately for the foreleg, midleg and hindleg. The TAP maximum length (L) and width (W) were the distances between the two setae tips that were furthest apart (between the most distal and most proximal setae, and between the two most lateral setae) (Fig. 1). Assuming an elliptical shape of the area covered by setae, we calculated the TAP area (A) as:

$$A = \frac{L}{2} \times \frac{W}{2} \times \pi. \quad (1)$$

We counted the number of setae per TAP separately for the foreleg, midleg and hindleg and measured the length of three setae each in the basal, middle and distal part of the TAP, if accessible. We measured the length (L_s) and width (W_s) of the spatulate tips of the setae (Fig. 2) and calculated an elliptic setal area (A_s), the likely adhesive area, as:

$$A_s = \frac{L_s}{2} \times \frac{W_s}{2} \times \pi. \quad (2)$$

Statistical analysis was carried out using the statistics package R (<http://www.R-project.org/>). In addition to descriptive statistics, mean comparisons by t -test and simple linear regressions, we used a linear mixed effects model to assess whether the direction in which a bedbug was facing influenced the force generated, using the direction (backward, forward, sideways) as a random factor within an individual. The within-individual variation of forces was graphically displayed. Because the intra-individual variation was

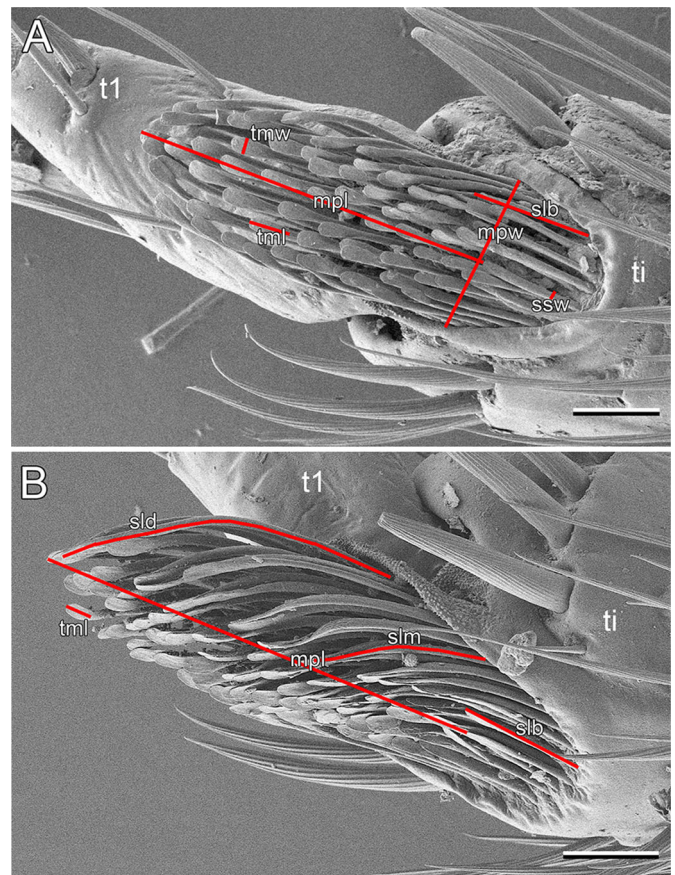


Fig. 1. Ventral and lateral aspect of the hairy tibial attachment pad (TAP) in adult *Cimex lectularius* and definition of the tibial and setal dimensional parameters. (A) Female foreleg. (B) Male foreleg. Note the gradient in the length of the setae, increasing distally. mpl, maximum pad length; mpw, maximum pad width; slb, setal length at the basal pad; tmw, maximum setal tip width; tml, maximum setal tip length; ssw, setal shaft width; slm, setal length at the middle part of the pad; sld, setal length at the distal pad; t1, first tarsomere; ti, tibia. Scale bars: 20 μm .

large, statistical analyses using repeated measurements proved uninformative of the attachment forces. Means and variances of the maximum forces per individual were analysed instead. Fisher's exact tests were used to examine difference in frequencies.

RESULTS

Attachment forces

Attachment on smooth resin

In our sample population, unfed females weighed 17% more than unfed males (Table 2). On the surface of the rotating drum, bedbugs slid to the outer edge until losing their foothold (Fig. S1). Under this condition, we expect friction to be the main contributing force to attachment generated by shearing of bedbug tibial tenent setae in adults and membranous pads in nymphs sliding outwards from the horizontal centrifuge drum. Attachment forces did not vary systematically with consecutive runs in males, females or nymphs (Fig. S2), or with the direction the individual faced on the drum (GLM; slope comparison forward versus backward position to the direction of drum rotation: $t=0.12$, $P=0.91$; sideways versus backward position to the direction of drum rotation: $t=0.33$, $P=0.75$). Subsequently, we only considered the maximum attachment force of 10 runs and we disregarded the direction in which the bug was facing.

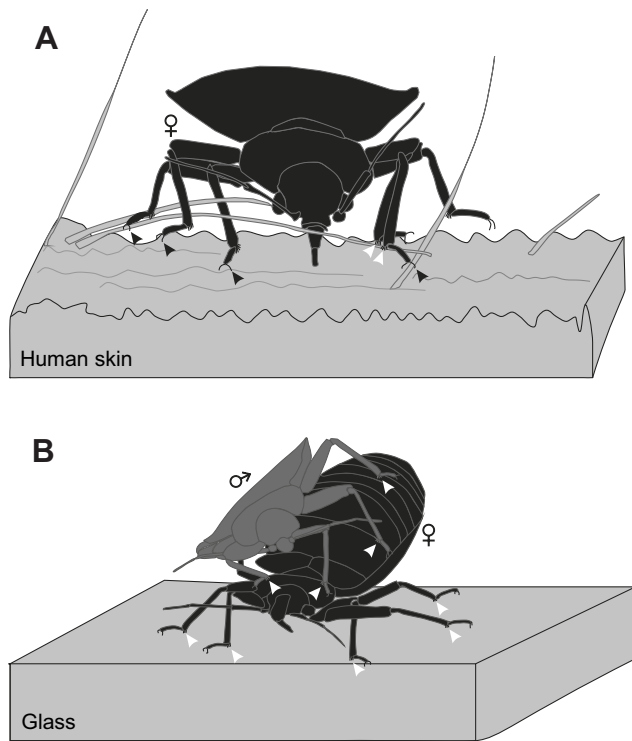


Fig. 2. Schematic diagram summarising observations on the position of the feet in male and female *C. lectularius*. Bedbugs attached to human skin (A) and to glass and female integument (B). White arrowheads point to tenent setae (tibial attachment pad, TAP) in contact with the substrate. Black arrowheads point to claws interlocked with human hairs and skin folds.

The maximum attachment force out of 10 runs differed between males, females and nymphs (Fig. 3, Table 2; one-way ANOVA, $F=27.61$, d.f.=2, $P<0.001$). On average, females generated a 10-fold higher force than nymphs, and males generated a force double that of females (Fig. 3, Table 2; t -test males versus females, $t=4.47$, d.f.=15, $P<0.001$). Within the sexes, there was no correlation between body mass and maximum attachment (males: Spearman's $\rho=0.071$, $N=8$, $P=0.882$; females: Spearman's $\rho=0.33$, $N=8$, $P=0.428$).

Attachment on hydrophilic and hydrophobic glass

Attachment forces varied from near-zero to 8.98 mN on the hydrophobic surface, and 0.75 to 8.29 mN on the hydrophilic surface. Four out of five males attached more strongly to the hydrophobic than to the hydrophilic surface whereas only one out of

four females did. However, this trend was within the range of chance (Fisher's exact test, $P=0.206$). Comparing forces within individuals on each of the surfaces, we calculated the ratio of maximum hydrophilic force to maximum hydrophobic force. It ranged from 0.17 to 3.36, excluding the near-zero outlier. The mean was 1.86 for females and 0.84 for males. Thus, on average, females attached more strongly to the hydrophilic surface, and males attached more strongly to the hydrophobic surface.

These observations suggest substantial dimorphism in attachment force in the direction predicted by sexual selection, supported by the predicted tendency of attachment to hydrophilic and hydrophobic surfaces. We next examined the context in which TAPs are used by males and females.

Use of TAPs during mating and feeding

Mating

Cryo-SEM imaging showed the TAP of the male left midleg and hindleg adhered to the female at the moment of fixation (Fig. 4A). The spatulate terminals of the TAP setae formed a tight contact with the cuticular surface of the female (Fig. 4D). Neither of the two males fixed during mating used their claws to grasp the female. Instead, the claws were lifted up and held off the female's body surface (Fig. 4B). However, on filter paper, claws of males and females interlocked with the fibrous substrate (Fig. 4A,C). These observations are not artefacts of fixation. When observed alive during copulation, all males had the TAP of all three left legs and one right leg in contact with the female dorsal cuticle surface during mating ($n=4$). If males anchored to the ground during copulation (which was not always the case; Fig. 2B), they never used TAPs and only claws of right legs.

Feeding

While attempting to feed, adults of both sexes and nymphs only sometimes had their TAPs in contact with smooth plateaus of human skin folds, and only of some legs (Fig. 2A): in females, 1–3 legs (sometimes 4 or 6 legs) ($n=6$); in males, 0–3 legs (only once for all 6 legs) ($n=6$); and in nymphs, 0–6 legs ($n=6$).

While sucking blood, the paired tarsal claws were always in contact with the human skin (Fig. 2) and this was true for all six legs of males, females and nymphs ($n=10$ observed for each). The claws were usually held oppositely spread and interlocked with skin folds or hairs upon contacting human skin.

These results suggest that TAPs are used by males to attach to females, and only sometimes contact the host skin. We next examined TAP morphology and asked which aspects are correlated to patterns of attachment.

Morphology

General description of the TAP

Our cryo-SEM images confirmed the absence of TAPs in nymphs (Kim et al., 2017; compare Figs 5, 6). The fleshy, smooth membrane that instead surrounded each tibio-tarsal joint (Fig. 6) was ventrally sculptured with miniature 'knobs' of ca. $1.3\text{--}1.7\text{ }\mu\text{m}^{-2}$ (1st instar) to ca. $0.3\text{--}0.5\text{ }\mu\text{m}^{-2}$ (5th instars) in density (Fig. 6A,B). Remnants of these knobs were also found in adults (see description below). Although not organised into TAPs and not possessing the spatulate terminals that setae of adults do, fifth (but not earlier) instars carried ca. 10 setae on each distal tibia (Fig. 6H–J).

The oval-shaped arrays of TAPs in adults covered with tenent setae were surrounded by fleshy tissue bearing knobs on each ventro-distal tibia and varied in length ($62\text{--}153\text{ }\mu\text{m}$), width ($32\text{--}54\text{ }\mu\text{m}$) and area ($1803\text{--}3680\text{ }\mu\text{m}^2$) (Fig. 5, Table 3). The legs also varied in setal

Table 2. Body mass, attachment force and safety factor of *C. lectularius* used in the current study

Parameter	Stage/ sex	N	Mean \pm s.d.	Minimum	Maximum
Body mass (mg)	Male	8	2.83 \pm 0.42	2.29	3.61
	Female	8	3.32 \pm 0.64	2.42	4.59
	Nymph	4	2.41 \pm 0.56	1.70	3.01
Friction force (mN)	Male	8	5.59 \pm 1.57	3.16	7.63
	Female	8	2.61 \pm 1.11	1.35	4.54
	Nymph	4	0.25 \pm 0.36	0.06	0.79
Safety factor	Male	8	182.83 \pm 62.99	83.50	303.00
	Female	8	79.44 \pm 32.30	45.30	138.00
	Nymph	4	8.81 \pm 11.68	2.09	26.30

Safety factor was calculated as attachment force/(body mass \times gravitation). See Fig. 3 for statistical analysis.

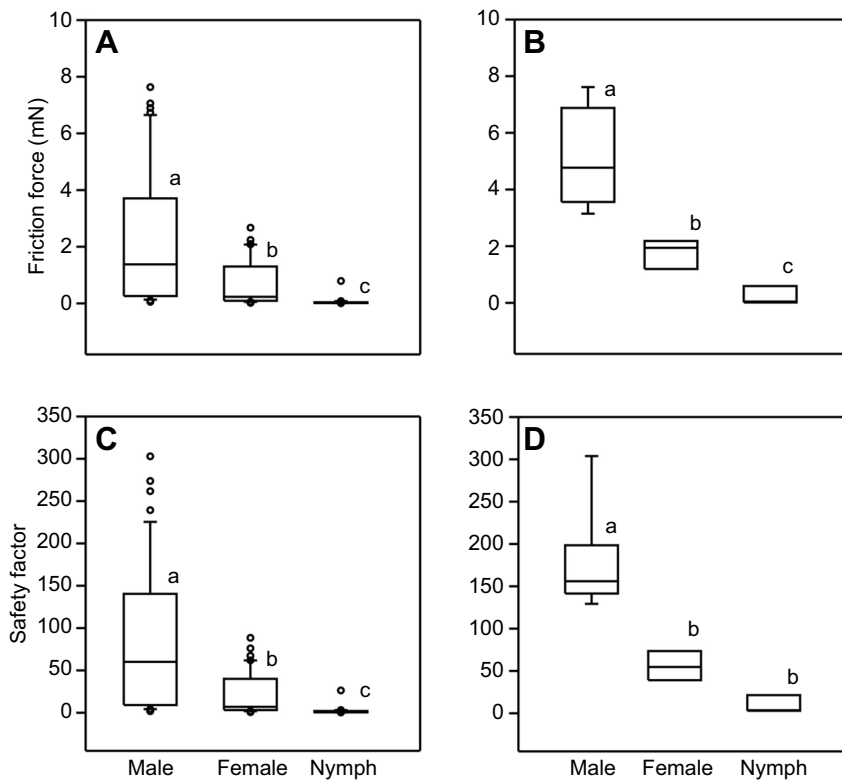


Fig. 3. Attachment forces (top) and safety factors (bottom) obtained in the centrifugal force experiment on a smooth epoxy resin surface. (A,C) Mean values. (B,D) Maximum values. Different letters indicate significant differences between females, males and nymphs (Kruskal–Wallis one-way ANOVA on ranks, all pairwise multiple comparison procedure, Dunn's method; A: $H_{2,103}=50.3$, $P\leq 0.001$; C: $H_{2,103}=49.1$, $P\leq 0.001$; B,D: $H_{2,19}=17.2$, $P\leq 0.001$). See Table 2 for means \pm s.d. and calculation of the safety factor.

number and TAP dimensions but showed several differences that are consistent between the sexes (Table 3; see below). Several TAP areas displayed blue autofluorescence upon UV excitation, particularly the tips of the setae and leg joints (Fig. 5E).

Tenent setae of the hairy TAP

The setal shaft length tended to decrease from the anterior to the posterior part of the TAP, the latter shafts being half as long as the

former (Fig. 5, Table 3). The setae possessed flattened spatulate terminal elements (Table 3) that were corrugated (Fig. 5L). Multiplying the area of the setal terminals (the adhesive contact area) by the number of setae resulted in estimated contact areas of 11.4–25.8 μm^2 per leg (on average 17.3 μm^2 , $n=29$) (Fig. 5D,F–K, Table 3). Detached TAPs left a pattern of distinct fluid droplets (footprints) on the substrate (Fig. 5N), similar in arrangement to the tenent setae in the TAP.

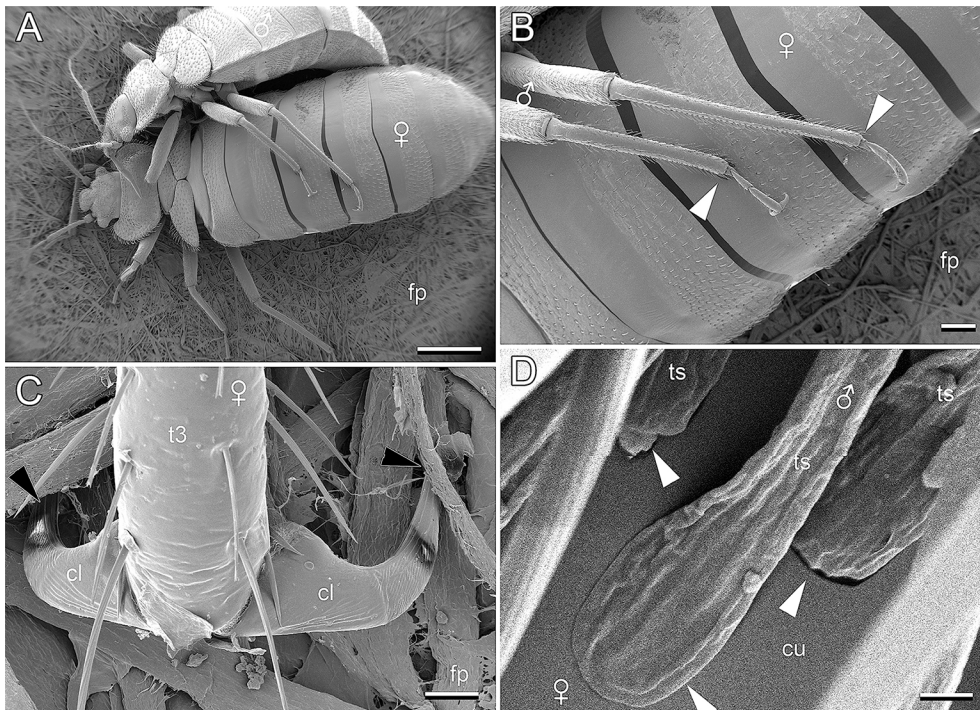


Fig. 4. Cryo-scanning electron microscopy (cryo-SEM) imaging of attachment sites of *C. lectularius* during mating. (A) The female is anchored to the substrate (filter paper, fp) by claws. The male attaches its left midleg and hindleg to the female's abdomen. (B) Detail of A; white arrowheads point to the male's TAPs placed on the smooth cuticle of the female's dorsal abdomen. The claws are held off the substrate and are not used for grasping the female. (C) Detail of interlocked claws (cl) of the female with the fibrous network of the filter paper substrate (indicated by black arrowheads). (D) The setal spatulate terminals of the TAP forming an intimate contact with the female's epicuticular surface. t3, 3rd tarsomere; ts, tenent setae. Scale bars: A, 1 mm; B, 200 μm ; C, 20 μm ; D, 2 μm .

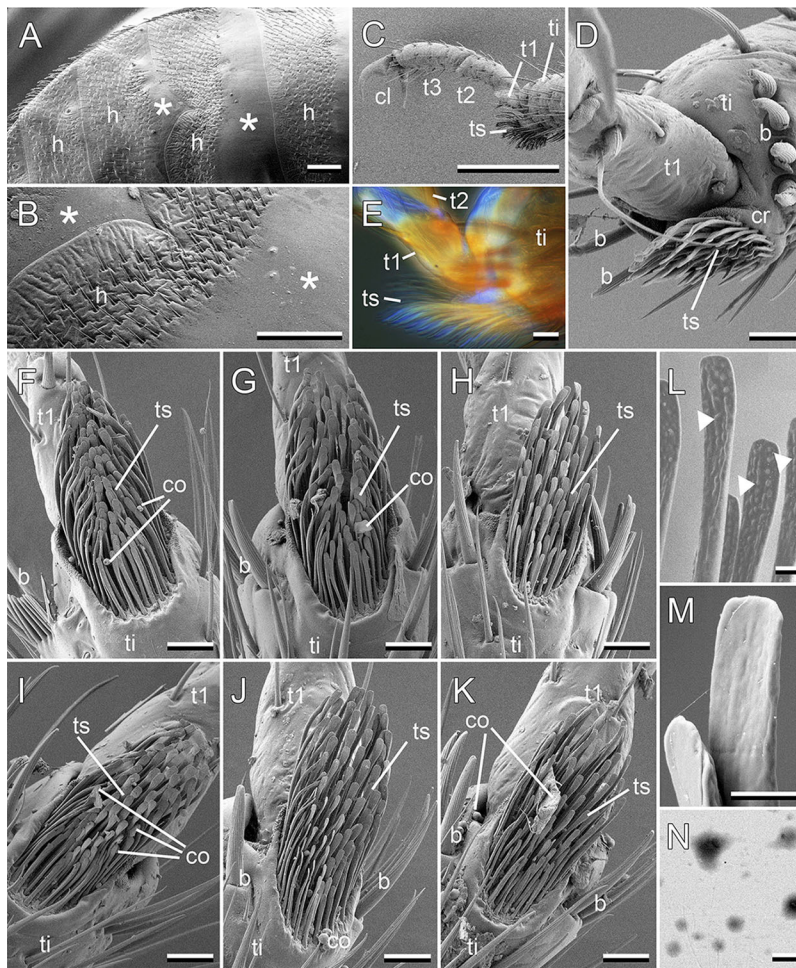


Fig. 5. Dorsal abdomen, tarsus and attachment pads in adult *C. lectularius*. (A) The substrate for the male attachment system during copulation: the epicuticular surface of a female abdomen, covered with transversal setal 'bands' (h) and smooth 'bands' (asterisks) in between. (B) Detail of A. The smooth bands occur in engorged, expanded females and were observed to be predominant attachment sites of male tibial TAPs bearing tenent setae. (C) Ventro-lateral view of a female foreleg tibia and tarsus. (D) Detail of the tibio-tarsal joint of a female foreleg. (E) Fluorescence light microscopy image indicating the presence of resilin in the tips of the tarsal setae and in the membranous areas surrounding intersegmental connections (blue). (F–H) Detail of a female TAP bearing tenent setae on the foreleg (F), midleg (G) and hindleg (H). (I–K) Detail of a male TAP bearing tenent setae on foreleg (I), midleg (K) and hindleg (K). (L,M) Details of terminal tenent setae: dorsal view (L), ventral view (M). Arrowheads point to the corrugated dorsal surface of setae terminal spatulae. (N) Footprints left after the detachment of tenent setae from a smooth, gold-covered substrate. b, bristle; cl, claw; cr, cuticular ridge; co, contamination; h, hairy band; t1–t3, tarsomeres 1–3; ti, tibia; ts, tenent setae. Scale bars: A–C, 200 μ m; D–K, 20 μ m; L–N, 2 μ m. All images are cryo-SEM micrographs except that in E.

Sexual dimorphism in TAP morphology and tenent setae

Our sample size is insufficient to statistically fully compare all six sex \times leg combinations for the 17 TAP and setal characters (Table 3). Conservatively using non-overlapping standard deviations as indicators of sexual differences, the main trends for sexual differences can be summarised as follows: (a) the number of setae per unit TAP area tended to be higher in males than in females, (b) foreleg TAP areas were larger in females than in males even though males had their largest and most setose TAPs on the foreleg, whereas in females, the midleg TAP was the largest and most setose (Table 3). In males, TAP area decreases towards the hindleg (although there was no clear trend in setae number per unit area). (c) The setae in the posterior part of the TAP were similar in dimension between males and females whereas those of the anterior part were markedly longer in males. (d) There was a positive relationship between spatula width (W_S) and length (L_S) in females (linear regression, $W_S = 1.34 + 0.29 \times L_S$, adjusted $R^2 = 0.28$, $P < 0.02$), but not in males ($P = 0.79$).

These results show a much lower attachment force in nymphs, correlated to an absence of TAPs and spatulate setae. In adults, a greater setae density and a steeper length gradient of setae in males than in females were correlated to differences in attachment force.

DISCUSSION

Previous suggestions for a sexually selected functional morphology of attachment devices were derived from a male-limited presence or male-specific geometry of attachment structures, or the observation

that beetle males adhered to females during mating and mate guarding (Aiken and Khan, 1992; Bergsten et al., 2001; Voigt et al., 2008, 2012, 2017b, 2019; Karlsson Green et al., 2013; Gloyne et al., 2014). Larger attachment forces may benefit males in withstanding dislodging attempts by defensive body flicking movements of females (Reinhardt et al., 2009) or competing males (Schanz, 1953; Pelletier and Smilowitz, 1987; Voigt et al., 2008, 2009). In the bedbug *C. lectularius*, we found several lines of evidence consistent with *a priori* predictions for a sexually selected function of the TAP, but with limited support for a naturally selected function. The evidence and their implications for future attachment studies are discussed below, as are intraspecific variation and its implications for control.

Sexual dimorphism in attachment

In situ fixed mating bedbug males had TAPs that were tightly adhered to the females, which supports existing suggestions for their function (Wigglesworth, 1938; Kim et al., 2017; Baker and Goddard, 2018). We did not find any evidence that claws interlock with the female's body, suggesting that the tenent setae on the male TAPs have a major role in adhering to females during mating. TAPs were used on host skin as well. However, the significance of such a naturally selected function is probably small given that nymphs obtain their blood meals but do not possess TAPs. A naturally selected function of, for example, carrying blood meals, could still occur within the sexes separately. However, we did not find that body mass, a predictor of blood meal size, was related to the maximum attachment force.

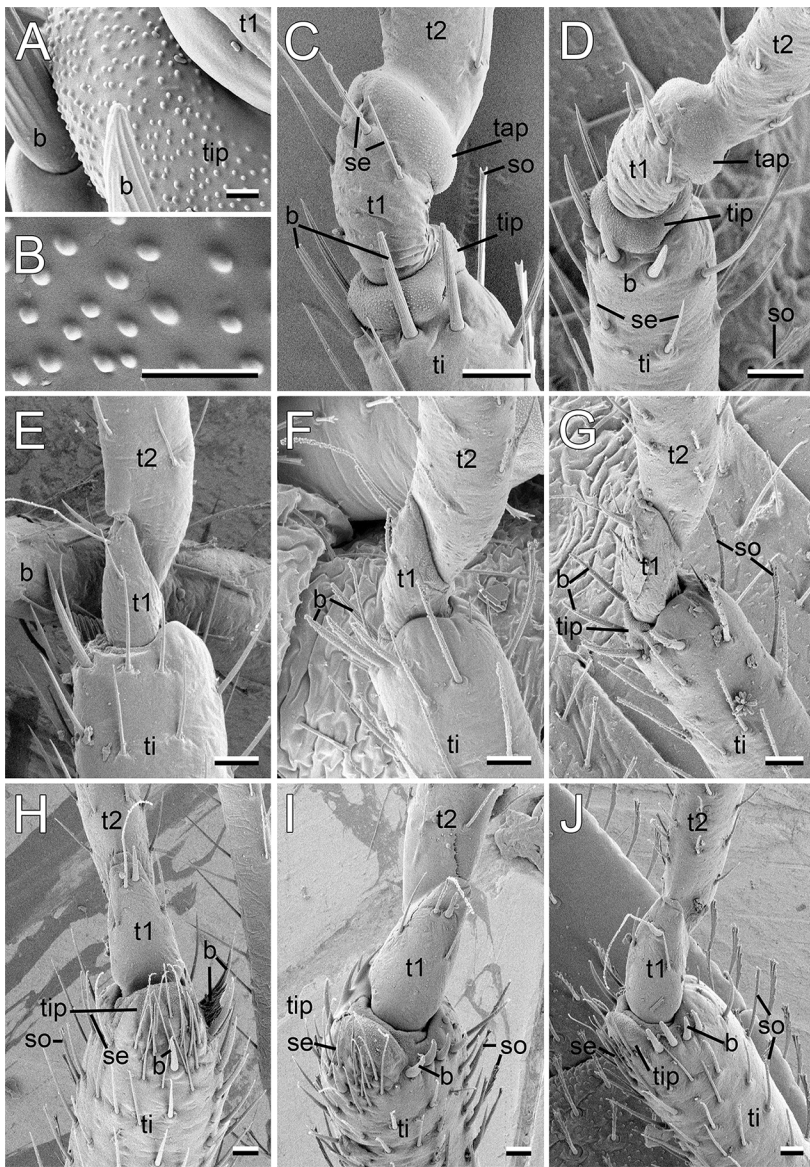


Fig. 6. The tibio-tarsal joint area in nymphal *C. lectularius*. (A,B) Detail of the knobby tibial pad surface on the 3rd leg of an L1. (C) L1, 2nd leg. (D) L1, 3rd leg. (E) LIII, 1st leg. (F) LIII, 2nd leg. (G) LIII, 3rd leg. (H) LV, 1st leg. (I) LV, 2nd leg. (J) LV, 3rd leg. b, bristle; se, seta; so, solenidia; t1–t3, tarsomeres 1–3; tap, tarsal pad; tip, tibial pad. Scale bars: A and B, 2 μ m; C–J, 20 μ m. L1, LIII and LV refer to first, third and fifth instar nymphs, respectively. All images are cryo-SEM micrographs.

Currently it is unclear whether the TAPs in the Cimicidae and Reduviidae are homologous or have evolved independently (Weirauch, 2007). Therefore, it also remains unclear whether TAPs are evolutionarily reduced in cimicid nymphs or have independently evolved in adult cimicids only. The observation that females of another cimicid species (*Haematosiphon inodorus*) do not possess TAPs at all (Lee, 1955) makes the evolutionary reduction of TAPs somewhat more likely.

The sexual difference in the number of setae per unit area was also consistent with larger attachment forces in males, rather than representing a sexual difference in body mass. Other indicators that selection on attachment structures was divergent between the sexes were the different relationship between spatula width (W_s) and length (L_s) in females and males as well as the setal length gradient along the TAP. Future studies should ask whether females possess TAPs because of a small additional benefit of attaching to the host skin during blood sucking or because the shared genome between the sexes prevented a complex trait like attachment pads becoming sex limited (albeit sex limitation has occurred in other species; Lee, 1955; Stork, 1981). Future studies should also look at how much of the TAP and setal variation

between the sexes contributes to doubling the attachment forces in males compared with females.

Another sexual dimorphism was observed in the gradient of setae length along the TAP (Table 3). How the marked extension of setae in the anterior part of the TAP in males may be related to the sexually selected function of the TAPs remains to be seen. Voigt et al. (2017b) showed that the gradient in setal length within the adhesive pad affected the shear angle and thereby the grasp strength in the mating position in rosemary beetles. It is not unlikely that a synergism between the setal gradient in adhesive pads and leg kinematics during mating also exists in bedbugs.

Finally, it would be interesting to study whether, in addition to its effects on setal size, selection also acts on setal material properties. For example, resilin incorporation renders cuticular structures soft and elastic with a high tensile strength, and resilin has been detected in the adhesive setae in insects (Niederegger and Gorb, 2003; Peisker et al., 2013) by autofluorescence emission at 420 nm after UV excitation. We report a similar autofluorescence signal in the TAP of bedbugs (Fig. 5E) and future work should define its role in attachment and clarify the resilin identity by examining pH-related changes in autofluorescence (Burrows et al., 2008) or genetic targeting.

Table 3. Description of the tibial attachment pad (TAP) in *C. lectularius*, separately for males and females and their different legs

Trait	Females			Males		
	Foreleg	Midleg	Hindleg	Foreleg	Midleg	Hindleg
TAP max. width (μm)	37.81 \pm 4.69 (3)	42.93 \pm 3.71 (5)	39.85 \pm 2.76 (5)	47.50 \pm 3.53 (5)	43.10 \pm 1.09 (3)	32.14 (1)
TAP max. length (μm)	86.92 \pm 7.64 (6)	94.12 \pm 6.13 (8)	79.74 \pm 9.97 (5)	83.49 \pm 7.94 (8)	89.67 \pm 11.67 (9)	78.16 \pm 8.29 (7)
TAP area (μm^2)	2498 \pm 370 (3)	3213 \pm 455 (5)	2472 \pm 424 (4)	3144 \pm 242 (4)	2942 \pm 125 (3)	1803 (1)
TAP area (% of foreleg)		134.2 \pm 8.6 (4)	96.4 \pm 15.5 (3)		87.6 \pm 7.7 (2)	57.7 (1)
Setae number	91.7 \pm 19.4 (7)	97.2 \pm 12.8 (8)	60.9 \pm 11.5 (7)	116.7 \pm 13.7 (7)	97.3 \pm 9.3 (7)	75.7 \pm 24.5 (7)
Setae number (% of foreleg)		117.9 \pm 29.6 (8)	74.0 \pm 23.0 (7)		85.3 \pm 20.6 (7)	65.7 \pm 22.0 (7)
Setae number per unit area (mm^{-2})	30,720 \pm 8441 (3)	29,373 \pm 2267 (5)	25,929 \pm 3088 (4)	35,883 \pm 6216 (4)	32,285 \pm 4339 (3)	38,265 (1)
Setae density (% of foreleg)		97.6 \pm 20.5 (4)	92.7 \pm 22.2 (3)		118.5 \pm 32.3 (3)	134.4
Shaft length of anterior part of TAP	55.4 \pm 2.5 (2,4)	No data	42.8 (1,2)	66.0 \pm 9.7 (5,6)	60.3 \pm 5.1 (5,6)	57.1 \pm 6.2 (3)
Shaft length of mid-part of TAP				50.7 \pm 4.6 (6,8)	45.6 \pm 4.8 (7,8)	46.6 \pm 5.7 (5,8)
Shaft length of posterior part of TAP	24.1 \pm 3.44 (6,14)	25.2 \pm 2.89 (7,21)	26.11 \pm 1.45 (3,9)	26.43 \pm 1.84 (7,18)	26.02 \pm 2.53 (8,21)	25.9 \pm 3.2 (7,18)
Setal shaft width				1.69 \pm 0.12 (2,2)	1.63 \pm 0.42 (2,2)	1.64 \pm 0.04 (2,2)
Setal tip max. width	3.49 \pm 0.42 (7,21)	3.2 \pm 0.37 (7,21)	3.25 \pm 0.41 (7,21)	3.13 \pm 0.39 (5,15)	2.82 \pm 0.37 (3,11)	3.10 \pm 0.21 (4,10)
Setal tip max. length	6.85 \pm 0.87 (5,15)	6.86 \pm 0.98 (7,20)	6.66 \pm 0.81 (7,20)	6.48 \pm 0.76 (8,24)	7.08 \pm 0.67 (8,23)	6.43 \pm 0.49 (6,18)
Setal width (% of foreleg)		85.0 (1)	96 \pm 12 (6)		No data	110.1 \pm 18.9 (3)
Setal tip area (μm^2)	18.6 \pm 4.7 (5)	17.9 \pm 4.2 (6)	17.1 \pm 3.5 (7)	17.1 \pm 2.6 (5)	16.2 \pm 3.5 (3)	16.2 \pm 1.3 (3)
Setae thickness (μm)		No data		0.20 \pm 0.04 (4,8)		

Data represent the mean \pm s.d. with the sample size in parentheses. In setal traits, the sample size is given as (N , n), where N is the number of setae and n is the number of individuals.

Using stereomicroscopic and cryo-SEM observations, we revealed that the spatulate terminal elements of males closely contacted the female cuticular surface (Fig. 4D). In agreement with selection to attach to the lipophilic female cuticle (Lewis, 1962), we found a tendency for males to attach slightly better to hydrophobic than to hydrophilic surfaces. The fact that we found the reverse tendency in females could suggest that the naturally selected situation may be that individuals attach slightly better to hydrophilic surfaces.

Tarsal fluids are known to mediate insect attachment to various surfaces (e.g. Gorb, 1998; Federle et al., 2002). Here, we report the existence of such fluids released by bedbugs via their TAPs (Fig. 5N). Whether males and females differ in their TAP-released fluid and relative attachment performance on surfaces of a broad range of wettability would be highly relevant to recent theory and data showing that sexual selection often includes antagonistic interactions (and co-evolution between the sexes) (reviewed by Rice and Gavrillets, 2014). Applying this reasoning to the attachment system, an advantage to females (and hence selection for it) of reducing male attachment ability may explain why female potato beetles distribute a grease on their elytra that reduces male attachment by increasing surface lubrication and hydroplaning (Voigt et al., 2008, 2009, 2017b). Because the bedbug is a model system of sexually antagonistic selection (e.g. Reinhardt et al., 2003, 2009, 2014), it would be interesting to test whether sexual conflict over male attachment to the female exists. Given that the connection between male attachment structure and female cuticle appears only slightly less intimate than the connection between male and female genitalia, one may expect the attachment system to be a hotbed of evolutionary change. More specifically, we predict strong sexual co-evolution, and hence rapid evolutionary diversification, between the composition of the adhesion-mediating fluid released by adhesive pads (Fig. 5N) and an anti-adhesive function of female surface lipids.

Bedbug attachment performance by comparison

Compared with the more usual attachment forces of 30–70 times body weight (safety factor) in some insects using their adhesive pads (Walker et al., 1985; Gorb et al., 2001; Stork, 1980a; Voigt et al., 2008; Zurek et al., 2017), our recorded maximum (303-fold; Table 2) represents the upper end of the spectrum. A larger figure, 534-fold safety factor, was recorded for female ticks, *Ixodes ricinus*

(Acari, Ixodidae) (Voigt and Gorb, 2017). However, in this case, the attachment was not by tenent setae or TAPs but by smooth adhesive pads (Voigt and Gorb, 2017) and the figure would reduce to ca. 4 when considering the 135-fold increase in body mass during blood uptake. Hence, even unrealistically assuming that bedbugs would use only TAPs to attach to host skin (which we know they do not; see Results), this would still amount to safety factors for fully fed bedbugs of around 100. We, therefore, assume that the majority of this attachment force is required by males to withstand dislodging by females or by rival males, or by staying attached during long-term mate guarding.

It is also important to note that adhesive forces scale with the second power of the linear dimension, whereas mass scales with the third power (see also Varenberg et al., 2010). As the safety factor is linearly expressed as attachment/body weight, it will not represent the true non-linear relationship between attachment force and body mass. The greatest deviation from linear relationships will be at small body sizes and we, therefore, expect the strongest deviations of the linearly expressed safety factors in animals of small body weight, such as ticks and bedbugs.

Variation in attachment and implications for bedbug control

Although not the focus of our study, some of our results may have implications for bedbug control. Our observations agree with earlier observations on bedbug climbing behaviour, TAP and setal morphology, as well as attachment forces (Wigglesworth, 1938; Walpole, 1987; Kim et al., 2017; Hottel et al., 2015; Hinson et al., 2017) but also expose some differences. These may be related to methodology, in which case it is important to discuss their validity, or to biology, in which case the generality of the results may not be projected from single-population studies. We note that sexual selection should commonly lead to population differences in attachment forces.

For example, other populations have slightly shorter spatulae than ours did (Kim et al., 2017), or fewer setae on the hindleg (ca. 75, compared with ca. 45 in Kim et al., 2017; Baker and Goddard, 2018). Such variation must be considered when projecting from attachment experiments to the design of smooth-surface traps.

Kim et al. (2017) report attachment forces for bedbugs perpendicularly pulled off from the substrate (and therefore

predominantly adhesion forces) in the range of 45 μ N on normal glass. Our values, measured in the horizontal direction (likely to be predominantly corresponding to friction), were several orders of magnitude larger, on the mN scale. Such force differences may be important if bedbugs are able to use TAPs on the smooth surfaces of bedbug traps. Unlike Kim et al. (2017), we found a sexual difference in attachment. It remains to be tested whether a possible difference in the type of force (putative adhesion versus putative friction) or in the measurement device (modified balance in Kim et al., 2017, versus centrifugal device in this study) explains the large differences between studies.

Hottel et al. (2015) did not observe a statistically significant difference between male and female bedbugs either, but they measured traction forces of bedbugs freely walking horizontally over the substrate. Traction force includes friction between the legs and the substrate but also muscle output. In addition, bedbugs dynamically generate forces while freely walking whereas those horizontally attached to a rotating centrifuge drum keep a rather static body posture while sliding to the centrifuge edge. It would be interesting to see whether the maximum muscle pulling power is similar between males and females despite the adhesive system differing strongly, which may explain the lack of sexual differences found by Hottel et al. (2015).

Sex differences in attachment may also be obscured if intra-individual variation in attachment is large. Very few studies have examined this source of variation, which we found to be sizeable. Averaging intra-individual variation will inevitably obscure sexual differences and the maximum attachment forces that bedbugs are capable of. Until the reason for the large intra-individual variation is found, we advocate the use of the maximum out of 10 trials, rather than using the mean. Our study, along with that on leaf beetles bearing tarsal setae (Zurek et al., 2017), indicates that the use of 10 trials does not introduce a systematic bias in attachment force measurements by muscle fatigue, energy or secretion depletion, or learning.

Conclusions

We reveal the use of attachment organs during copulation in bedbugs and report sexual dimorphism in attachment forces to smooth surfaces. The pattern of variation between the sexes and the absence of the TAP in nymphs is consistent with the idea that important aspects of the attachment system currently function in the context of sexual selection. However, the TAPs are not exclusively used during mating and systematic screening of their use on other smooth surfaces is recommended for any biomimetic applications of bedbug control. We reiterate that the close contact of male attachment structures with female integumental surfaces makes it likely that the large diversity in attachment structures (Gorb, 2001; Beutel and Gorb, 2001) may be the result of similarly strong diversifying selection similar to that for male genitalia.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: K.R., D.V., S.G.; Methodology: K.R., D.V., S.G.; Validation: K.R., D.V.; Formal analysis: K.R., D.V.; Investigation: K.R., D.V.; Data curation: K.R., D.V.; Writing - original draft: K.R., D.V.; Writing - review & editing: K.R., D.V., S.G.; Visualization: K.R., D.V., S.G.; Supervision: S.G.

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Supplementary information

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