









# The exceptional attachment ability of the ectoparasitic bee louse *Braula coeca* (Diptera, Braulidae) on the honeybee

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## Abstract

Bee lice (Braulidae) are small parasitic flies, which are adapted to live on their bee host. As such, the wingless *Braula coeca* is a parasite of the common honey bee *Apis mellifera* and it is well adapted to attach to its hairy surface. The attachment system of *B. coeca* provides a secure grip on the fine setae of the bee. This is crucial for the parasite survival, as detachment from the host is fatal for the bee louse. The feet morphology of *B. coeca* is well adapted to the challenging bee surface, notably by strongly broadened claws, which are split into a high number of comb-like teeth, perfectly matching the diameter of the bee hairs. Based on microscopy observations, both the morphology and material composition of the tarsi of *B. coeca* are characterized in detail. Using high-speed video analysis, we combine the morphology data on the attachment system with a behavioural context. Furthermore, we directly measured the attachment forces generated by the bee lice in contact with the host. In particular, the claws are involved in attachment to the host, as the interstices between the teeth-like spines allow for the collection of several hairs and generate strong friction, when the hairs slip to the narrow gap between the spines. The overall morphology of the tarsus produces strong attachment, with average safety factors (force per body weight) around 1130, and stabilizes the tarsal chain with lateral stoppers against overflexion, but also allows for the fast detachment by the tarsal chain torsion.

## KEYWORDS

adhesion, biomechanics, ecomorphology, friction, interlocking, parasitism

## INTRODUCTION

Parasitism is a widespread phenomenon across the animal kingdom and it is very common among insects (Knipling, 1992). The parasite benefits from this interaction at the expense of the host, for example, by feeding on blood, or food collected by the host, which therefore often attempts to eliminate the parasite (Poulin, 1998; Schulte et al., 2010). This competition results in an evolutionary arms race

with reciprocal selective pressure, leading to counter-adaptations in both host and parasite (Schulte et al., 2010). Therefore, parasitic insects show more adaptations in morphology, reproduction or dispersal, the more host-specific they are (Poulin, 1998).

Within Diptera, generalists with a wide range of habitats, like mosquitoes, show only few alterations from the typical bauplan, whereas more specialized species display drastic changes in the morphology (Petersen et al., 2018). Parasitic flies in particular are usually highly specialized. For example, the deer ked *Lipoptena cervi* (Linnaeus, 1758) sheds its wings after finding a host (horses, deers, cattle), displays a dorso-ventrally

Thies H. Büscher and Dennis S. Petersen contributed equally to this study.

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flattened body with elongated legs and differently sized claws and pulvilli on each tarsus (Haarløv, 1964; Kaitala et al., 2009). The swift louse fly *Crataerina pallida* (Latreille, 1812) is strictly host specific, strongly dorso-ventrally flattened, possesses rudimentary wings only and has enlarged tridentate claws, to cling on bird feathers effectively, even during the fast acrobatic flight of its host, the common swift (*Apus apus* (Linnaeus, 1758)) (Petersen et al., 2018).

Reliable attachment is essential for highly specialized parasitic insects, often resulting in the loss or reduction of wings (Liu et al., 2019; Wagner & Liebherr, 1992). Normally, the attachment system of insects consists of either smooth or fibrillar attachment pads, claws and sometimes additional structures, such as for example an empodium (Beutel & Gorb, 2001; Friedemann, Schneeberg, & Beutel, 2014; S. N. Gorb et al., 2001). The attachment pads are used to attach to smooth surfaces due to van der Waals forces by getting into intimate contact with the surface and, hence, maximizing the contact area (Arzt et al., 2003; Persson, 2013; Persson & Gorb, 2003). The attachment pads also secrete liquids during contact formation for capillary adhesion (Drechsler & Federle, 2006; S. N. Gorb, 1998; Kovalev et al., 2013). If the surface gets rougher, claws are used to cling to the surface (S. N. Gorb, 2001). Both parts of the attachment system work synergistically (Song et al., 2016).

However, compared to smooth or slightly rough surfaces, strong and reliable attachment on host surfaces like feathers, hairs or skin is a challenging task, because proper contact formation to the substrate is hampered due to the complex topography of the surface and the very flexible nature of the host surface microstructures. The entire attachment process becomes even more difficult, if the host gets smaller, because the size difference between the parasite attachment system and the host surface structures is usually strongly reduced.

A species that successfully solved these problems is the enigmatic bee louse *Braula coeca* Nitzsch, 1818, a kleptoparasite of the honey bee *Apis mellifera* Linnaeus, 1758 (Figure 1a). Braulidae (including *B. coeca*) are nested within Ephydroidea, the *Drosophila* relatives, and form with Cryptochetidae a sister group to the Drosophilidae (Wiegmann et al., 2011). *B. coeca* with a size of 1–2 mm is completely covered with setae. It is apterous, even lacking halteres (Imms, 1942). Since the thorax is very short, the legs originating from it are comparably long in relation to the remaining body, and the insertion of the coxae is shifted to the centre of the body ventrally. Eyes are rudimentary, but a field of olfactory sensilla (Börner, 1908) is present, situated in a groove just at the base of the bristle-like antennae (Grimaldi & Underwood, 1986). In combination, head, thorax and abdomen form an ellipsoid, with the abdomen being the dominant tagma of the body. Besides the legs, nothing protrudes from the body.

Bee lice are considered only a negligible threat to their hosts and of no importance in apiculture (Haftu et al., 2015), but they are often confused with the Varroa mite *Varroa destructor* (Anderson & Trueman, 2000) (Anderson & Trueman, 2000; Ramsey et al., 2019), which is harmful for the bee colonies. Bee lice are oviparous and reproduction takes place in the beehive (Grimaldi & Underwood, 1986; Örosi-Pál, 1938). Younger drones and queens are more likely to be infested by bee lice in the beehive after mating (Strauss et al., 2014). Important to note is that the dispersion of bee lice might be connected to the old queen leaving

the beehive during swarming events, since bee lice preferably attach themselves to the queen (Rodrigues & Serrano, 2019; Strauss et al., 2014). Drifting workers and drones might play a role in the dispersal of *Braula* as well, especially drones, since they are readily accepted in other colonies (Free, 1958).

Without strong and reliable attachment to the bee's surface, *B. coeca* would not be able to infiltrate into new bee colonies or to sustain a healthy population. However, attachment to the surface of a flying insect can be difficult and requires special devices to remain on the hairy surface of the host. Some authors have already mentioned the specialized foot appendages found in bee lice (Alfonso & Braun, 1931; Argo, 1926; Grimaldi & Underwood, 1986; Skaife, 1922). Especially, the strongly broadened, comb-like claws are, to our knowledge, rather unique across hexapods. However, it is not clear, how this very special attachment system actually works and why such foot appendages may have evolved, to attach to bees. Therefore, the aim of this study was (1) to characterize the morphology and material composition of the attachment structures of *B. coeca* in detail with the help of scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) (Michels & Gorb, 2012), (2) to analyse the active attachment process and behavioural adaptations of the bee lice with the help of high-speed video analysis and (3) to directly measure the attachment forces, bee lice are able to generate, while they are on their host. The results are compared with the attachment force of other insects and shed light on the biomechanical requirements necessary to properly generate strong attachment on a complex surface topography like bee hairs (Thorp, 1979), while being able to easily detach from it. In addition, the study provides a guideline, on how to measure attachment forces of such small insects on their three-dimensional native substrates reliably.

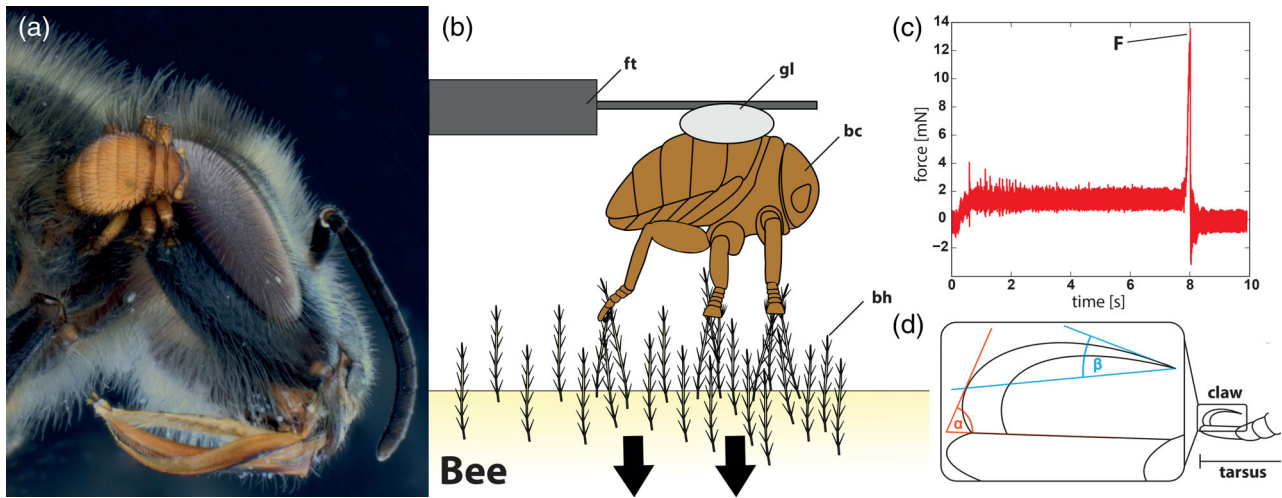
## MATERIALS AND METHODS

### Animals

Adult bee lice (*B. coeca*; Diptera, Braulidae) were collected during November 2018 from honeybee (*A. mellifera scutellata*; Hymenoptera, Apidae) colonies maintained at the experimental farm of the University of Pretoria, South Africa (25°45'11"S, 28°15'29"E). Together with 30–35 honeybee workers, 10 bee lice were stored in one polycarbonate cage with a size of 11.5 × 10 × 14 cm. The cage was equipped with a small piece of honeycomb (5 × 5 cm) and feeding vials for the bees. Tiny holes at the bottom enabled ventilation. Prior to experiments, the cage was stored in an incubator at 30°C and 45% relative humidity in darkness, simulating conditions within the hive (fig. 1 in Köhler et al. (2013)). The bee lice were kept at most 2 days in the laboratory before being used in the experiments.

### Scanning electron microscopy

Several adult bee lice, dissected single tarsi of all three leg pairs of the same species, as well as honeybees with bee lice attached to them,



**FIGURE 1** (a) The bee louse *Braula coeca* attached to the head region of its host, the honeybee *Apis mellifera scutellata*. (b) Experimental setup. The bee louse (bc) was attached to the tip of a strong needle which was mounted to a Fort25 force transducer (ft) and pulled off from the bee by actively pulling the bee away from the force transducer. (c) Representative force-time curve of the attachment force. (d) Schematic drawing of the measured base ( $\alpha$ ) and tip ( $\beta$ ) claw angles. bc, *B. coeca*; bh, bee hair; F, maximum pull-off force; ft, force transducer; gl, queen bee marking glue

were dehydrated in an ascending alcohol series. Afterwards, the samples were critical point dried using an automatic Leica EM CPD300 (Leica, Germany) and subsequently sputter-coated with a 10 nm layer of gold-palladium (Leica Bal-TEC SCD500). SEM images were obtained with a Hitachi TM3000 (Hitachi High-technologies Corp., Japan) at 15 kV acceleration voltage, using a rotatable specimen holder (Pohl 2010). Higher magnification images for details of the attachment system were obtained with a Hitachi S4800 (Hitachi High-technologies Corp.) at 5 kV acceleration voltage. All images were postprocessed, using Affinity Photo and Affinity Designer (Serif Ltd, United Kingdom) and subsequently, morphological aspects were measured using the software ImageJ (v2.0.0-rc-43/1.50e, Wayne Rasband, National Institutes of Health, USA). Distances within the images (e.g., interstices between claw tips etc.) were measured, using ImageJ's 'Straight Line' tool. Claw angles (e.g., bending angle of the claw, Figure 1d) were measured, using the 'Angle' tool.

### Confocal laser scanning microscopy

For CLSM analysis, tarsi of bee lice stored in ethanol (70%) were transferred into glycerine ( $\geq 99.5\%$ ) and mounted on a microscope slide. The autofluorescence of the samples was analysed using a Zeiss LSM700 confocal laser scanning microscope (Carl Zeiss MicroImaging GmbH, Germany), equipped with four stable solid-state lasers to assess the material composition. Following Michels and Gorb (2012), a 405 nm laser line in combination with a bandpass emission filter was used, transmitting 420–480 nm, for less sclerotized, potentially resilin-dominated, soft cuticle. Laser lines with values 488 and 555 nm together with long-pass emission filters transmitting light wavelengths equal to or bigger than 490 and 560 nm were used for detecting autofluorescence of more sclerotized cuticle. The 560 nm

long-pass emission filter together with a 639 nm line were used to detect autofluorescence exceeding this range (see fig. 1 in Büsse and Gorb (2018)). The signal of the autofluorescence was transformed to maximum intensity projections using the software packages ZEN2008 (Carl Zeiss MicroImaging GmbH), Affinity Photo and Affinity Design (Serife, West Bridgford, UK). One representative tarsus was visualized in the following to describe the relative differences in the material composition. The combination of the autofluorescence signals in the maximum intensity projections in single pixels allows for a qualitative estimation of the presence of different cuticle components (Andersen, 1979; Michels & Gorb, 2012; Vincent, 2002), but does not yield a quantitative measure of the precise stiffness of the cuticle.

The colours indicate the main signal of autofluorescence and enable an estimation of the material composition with regard to the relative degree of sclerotization in the attachment system. The colours correspond to following cuticle conditions: (1) Blue colours represent soft cuticle (2) green-like colours represent moderately sclerotized cuticle (3) red-like colours represent cuticle with higher degree of sclerotization. Consequently, a higher proportion of red colours corresponds to higher sclerotization.

### High-speed video analysis

High-speed video recordings of 12 living bee lice running on smooth glass surface and on freshly killed honeybees were produced. Special attention was given to the tarsal appendages of the bee lice. Locomotion was captured using a stereomicroscope (Olympus SZ51, Olympus, Japan) equipped with a Moticam 5.0 MP microscope camera (Motic). Videos were recorded at 150 frames per second and with a resolution of  $648 \times 486$  pixel. All experiments were conducted within 48 h after the bee lice had been captured. The attachment to the bee

is dominated by the presence of setal structures on the bee surface and, hence, the claws play a major role for attachment and locomotion. To compare the performance of the pulvilli without the contribution of claws, a smooth glass substrate was used to investigate the function. By this, we were able to estimate the interaction between the attachment system on a standardized substrate lacking microscopical structures. The (more or less) smooth surfaces within the bee hive, however, are most likely neither completely smooth, nor uniform in terms of surface texture or stiffness, and, therefore, were not used for the observation of the usage of the pulvilli.

## Force measurements of *B. coeca* on honeybees

Force measurements were performed to evaluate the maximum attachment force of bee lice on their hosts (Figure 1b). The force was captured using a BIOPAC MP 100 data acquisition system (BIOPAC System Inc, Goleta, California) by actively pulling bee lice off from honeybees in vertical direction. This was achieved by fixing the bee louse to a Fort25 force transducer (25 g capacity, <2 mg resolution; World Precision Instruments Inc., Sarasota, Florida). To separate a bee louse from its host, both were temporarily immobilized by cooling them down for 2 min at  $-20^{\circ}\text{C}$ . A strong insect pin (size 2, stainless steel) was glued to the thorax of a bee louse using queen marking glue (Human et al., 2013). The insect pin together with the bee louse was then mounted on the cantilever of the force transducer without allowing deflection of the insect pin. Prior to the force measurements, the bee lice were allowed to recover from the temporary immobilization. Then they were brought into contact with the hairs of the thorax of a freshly killed bee. When successful contact was made, the bee was pulled away in the vertical direction using forceps. Force-time curves were visualized with the software AcqKnowledge 3.7.0 (BIOPAC System Inc). The maximum attachment force was determined as the peak force observed before bee lice had been detached from the bee (Figure 1c). In total, 18 bee lice were tested three times each. After the force measurements were performed, all the bee lice were killed in a freezer and stored in ethanol for weight measurements. The weight of the bee lice was measured using a Sartorius ultramicrobalance MSE 2.7S-DM (Sartorius, Germany;  $\leq \pm 0.00025$  mg) after removing the lice from the ethanol and storing them on filter paper for 15 s. The measured attachment forces and masses are listed in Table S1.

## RESULTS

### Morphology of the bee lice attachment system

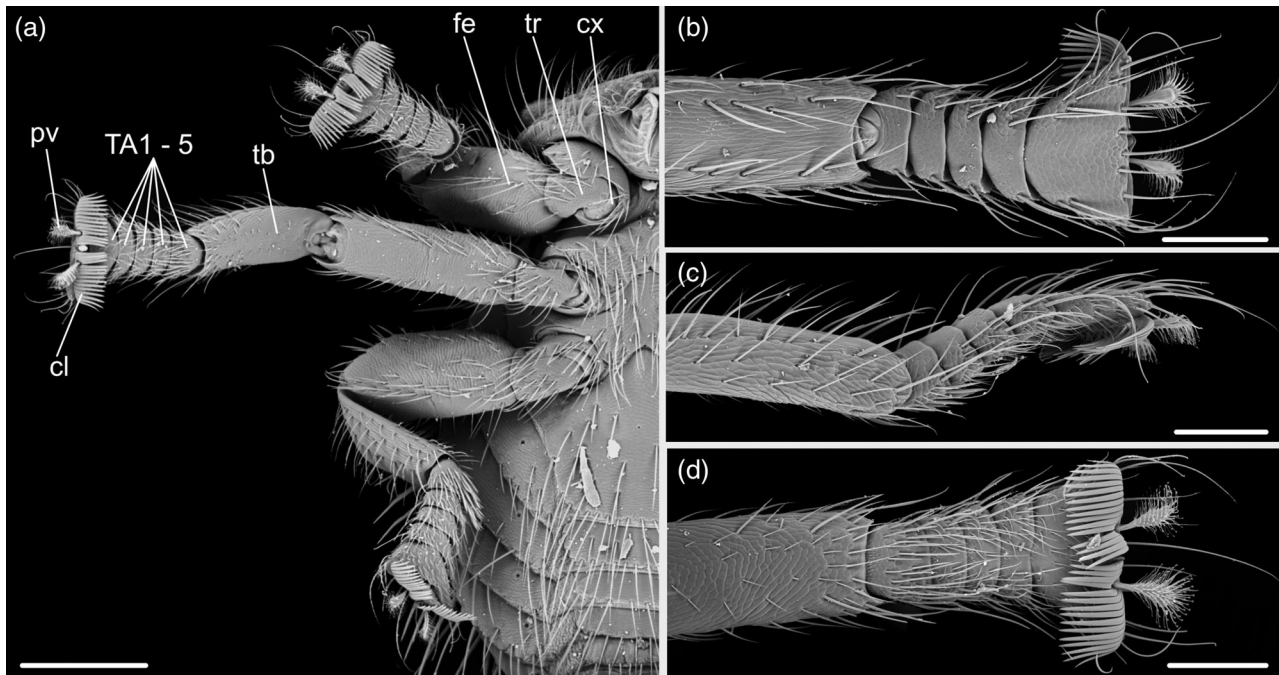
The tarsi of *B. coeca* consist of five tarsomeres (Ta1-5), each of them dorso-ventrally compressed and broadened from the base to the tip (Figure 2). The following anatomical designations refer to the position relative to the tarsus. The lateral edges of every tarsomere, as well as the tibia, are widened towards the tip (Figure 2a). The tarsus is hairless

dorsally, but covered with setae ventrally (Figure 2a). Patches of elongated setae are present at the lateral tips of the tarsomeres and a line of very long setae is present at the distal-most edge of the dorsal side of Ta5, protruding from the pretarsus. The attachment system is composed of two primary components: A similarly shaped pair of strongly subdivided claws and two pulvilli. An empodium is not present (Figure 2b-d). Each claw is comb-shaped with a single row and consists of 13 to 16 evenly spaced teeth. The teeth of the combs are distally pointed and broad based with an angle of  $64.4 \pm 5.7^{\circ}$  (mean  $\pm$  SD) at the base and an angle of  $16.2 \pm 3.7^{\circ}$  at the tip ( $n = 5$ ). Consequently, the interstices between the teeth get narrow towards the bases of the teeth. The distance (tip-to-tip) between the teeth is  $10.07 \pm 1.02$   $\mu\text{m}$  ( $n = 9$  for three different tarsi), while the interstice narrows down at the distance of 65  $\mu\text{m}$  towards the base (measured from the claw tip to the base) to  $0.99 \pm 0.33$   $\mu\text{m}$  gap width at the base ( $n = 9$  for three different tarsi). The interstice stays rather wide for the first 50  $\mu\text{m}$  and strongly narrows towards the base of the claw, until the teeth merge at the base. Close to the median line, the third, fourth and fifth medial teeth are curved, leaving a passage for the pulvillus (Figure 2d). The number of teeth per claw was neither constant between different individuals nor legs, nor always the same for the pair of claws of the same foot. Ranging from 13–16 teeth per claw (mean: 15,  $n = 3$ ), the area median of the pulvillus passage has approx. 4 teeth, leaving 9–12 teeth for the remaining area of the claw. The comb formed by each claw is curved in both horizontal and vertical directions. Most of the teeth are straight, but the contralateral outer teeth are slightly curved laterally to the tarsus (Figure 2d). Similar, the medial claw teeth are slightly curved towards the median line of the tarsus. The shape and location of the claw results in an inverted arrangement in comparison to most other insects: in *B. coeca*, the tips of the claws are directed towards the centre of the animal in unstrained posture.

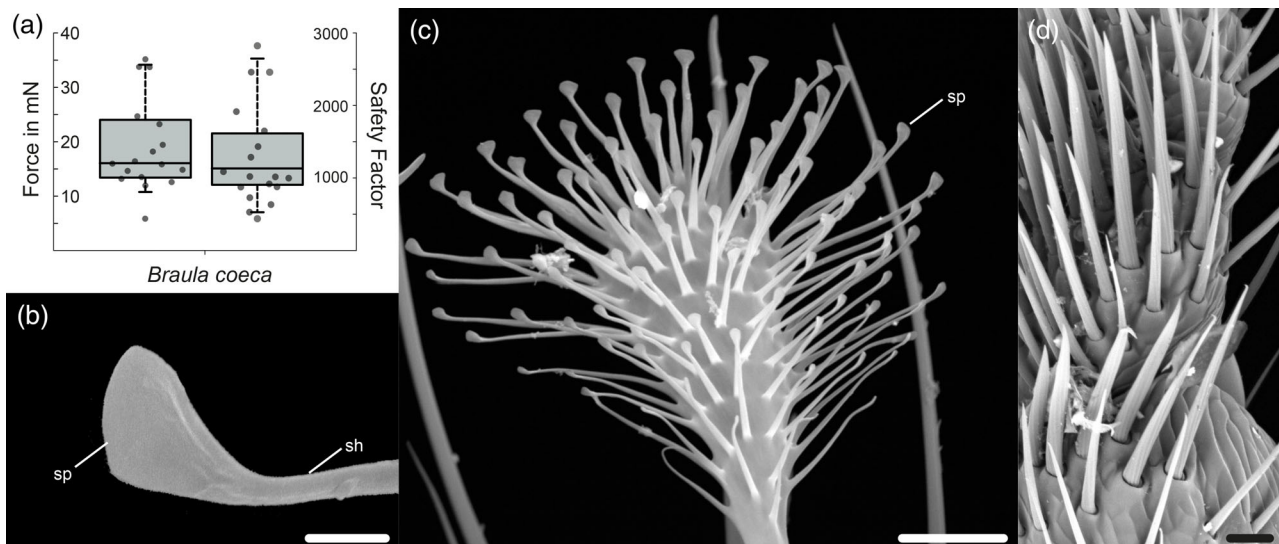
The pulvilli originate at the base of the claws and pass through the claws, as mentioned above (Figure 2d). The overall shape of each pulvillus is elliptic and similar for each leg pair (Figure 2a). These fibrillar attachment devices hierarchically split from the base to the tip into several setae. At the tip, each seta is terminated with an elliptic spatula. Each pulvillus consists of approx.  $132 \pm 15$  setae ( $n = 6$ ; both pulvilli counted on one pro-, meso- and metathoracic leg of each animal, respectively), with an approximate adhesive area of  $6.93 \pm 0.45$   $\mu\text{m}^2$  per seta ( $n = 3$ , Figure 3b). This results in a projected spatula area of  $914.32 \pm 103.5$   $\mu\text{m}^2$  ( $n = 6$ ; both pulvilli counted on one leg per thoracic leg pair of each animal respectively) per pulvillus, with a density of  $0.09 \pm 0.01$  setae per  $\mu\text{m}^2$  ( $n = 14$  pulvilli from seven different legs). The shafts of most setae are rather similar in diameter, but the shafts of central setae are shorter than those of setae located at the edge of the pulvillus. Due to the shape of the pulvillus base, the length of the setae results in a rather homogeneous distribution of spatula projection. As the pulvilli pass through the claws, this conformation results in the extrusion of pulvilli from the distal-most edge of the claws and in the inward oriented claw tips (Figure 2).

Regarding the attachment system, no differences between different thoracic leg pairs were observed, except a patch of grooming setae found on the metathoracic tarsi. They are situated ventrally on





**FIGURE 2** Scanning electron microscopic images showing the attachment structures of *Braula coeca*. (a) Ventral view of right half of the body. (b–d) Details of the tarsus. (b) Dorsal view. (c) Lateral view. (d) Ventral view. Scale bars: 250  $\mu\text{m}$  (a), 100  $\mu\text{m}$  (b–d). cl, claw; cx, coxa; fe, femur; pv, pulvillus; tb, tibia; tr, trochanter; TA1–5, tarsomeres



**FIGURE 3** (a) Attachment forces of *Braula coeca* on the bee. Boxplot showing the attachment force and respective safety factors (pull off force divided by the weight force) prior to detachment of *B. coeca* from its host *Apis mellifera scutellata*. In total, 18 individual bee lice were measured. (b–d) Scanning electron microscopic images of details of tarsal structures. (b) Tip of pulvilli setae. (c) Pulvillus. (d) Grooming setae. Scale bars: 1  $\mu\text{m}$  (b), 15  $\mu\text{m}$  (c), 10  $\mu\text{m}$  (d). sh, shaft; sp, spatula

the posterior part of the first and second tarsomere, as well as on the apical tip of the tibia. Approximately  $42 \pm 2$  setae can be found in those grooming patches ( $n = 3$ , from three different animals). The setae in the outer region of the patch (Figure 3d) are shorter and thinner (diameter at the widest point =  $3.43 \pm 0.7 \mu\text{m}$ , length =  $40.63 \pm 4.44 \mu\text{m}$ ;  $n = 5$ ), the inner setae are longer (diameter at the widest point =  $5.31 \pm 0.31 \mu\text{m}$ , length =  $62.57 \pm 2.47 \mu\text{m}$ ;  $n = 5$ ).

### Analysis of the attachment posture of the bee lice on honey bees

Attachment sites of adult bee lice attached to the thorax of their hosts were analysed (Figure 4a). In general, the legs were bent and positioned more towards the thorax of the bee louse. The tarsi were aligned parallel to the host's exoskeleton and the claws were folded

inwards, so that the claw tips were pointed towards the thorax of the bee louse (Figure 4b,c). Figure 4b shows a claw that has been pulled through the honey bee hairs before fixation. The hairs were well-ordered perpendicularly to the claw base and interlocked within the clamping regions of the claw teeth. Figure 4d displays the size relationship between a single clamping region and bee hair in more detail. The diameter of a bee hair (3.4–5.3  $\mu\text{m}$ ) fit to the available space between two claw teeth at the distal end ( $10.07 \pm 1.02 \mu\text{m}$ ,  $n = 9$  for three different tarsi). Therefore, bee hairs became ordered on top of each other during the interlocking process as seen in Figure 4e. According to our observations, the pulvilli did not play any role during the attachment in regions densely covered with hairs (Video S1).

### Material composition of the cuticle of bee lice attachment system

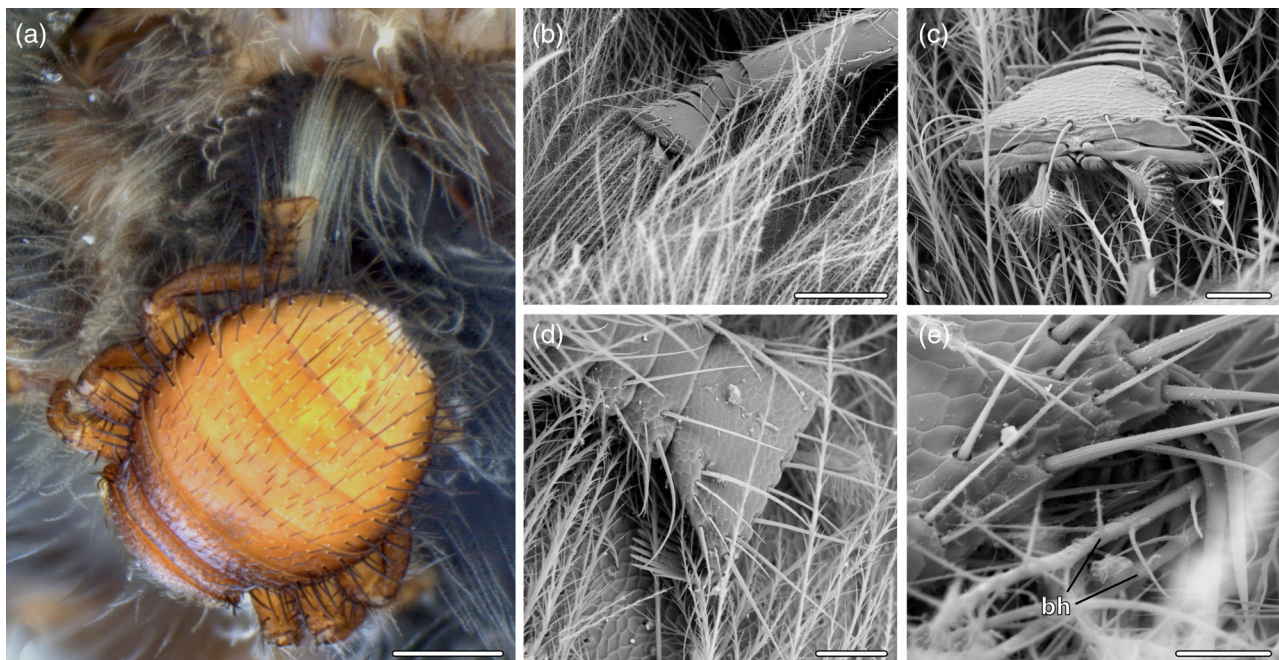
The major part of the tarsus is moderately sclerotized. The dominant regions with the highest degree of sclerotization are the bases of the claws and the median ventral region of the tarsomeres (Figure 5). The bases of all setae, especially in the grooming setae of Ta1, are considerably sclerotized as well. The grooming setae are generally more sclerotized than the remaining setae and hence, presumably, stiffer than the remaining ones. However, all setae exhibit a gradient of stiffness from base to tip, with a more sclerotized base and a softer tip, as well as a soft socket. Besides the strongly sclerotized median ventral area, the tarsomeres reveal large patches of soft, less sclerotized cuticle laterally, indicated by the blue signal in the CLSM maximum intensity projection (Figure 5a). These lateral patches form a continuous

ridge along the length of the tarsus (Figure 5b). The bases of the pulvilli are dominated by a blue autofluorescence signal as well, and, presumably, are very soft. The adhesive setae, originating from the base of spines, show a green signal at the shaft and the spatulas show a blue signal: There is the stiffness gradient from the stiff base to the soft tip. The claws are strongly sclerotized at the base (red signal) and slightly less sclerotized towards the tip.

### Locomotion of *B. coeca*

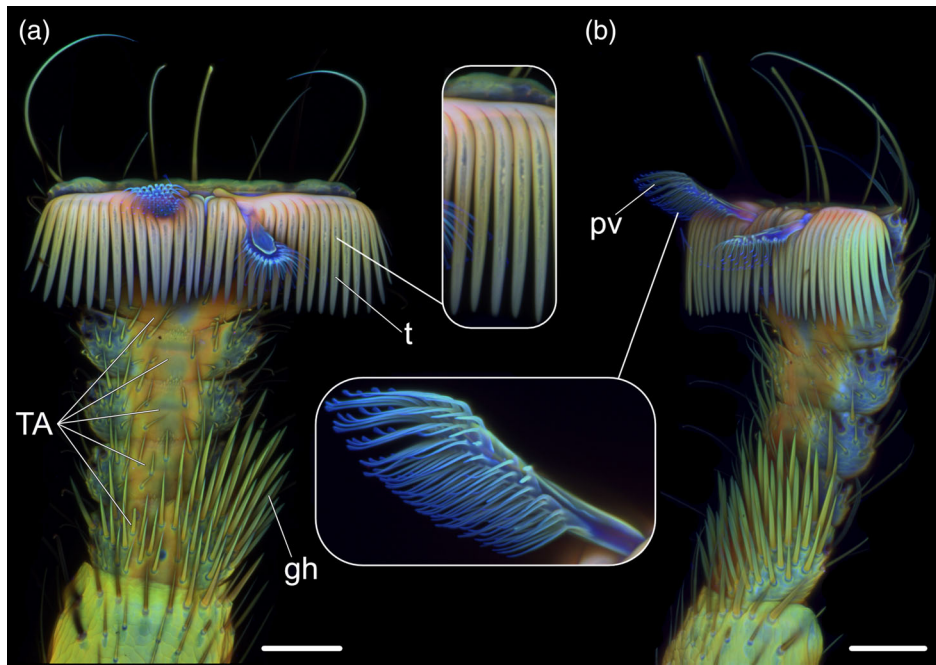
The bee lice (*B. coeca*) can freely, fast and efficiently move in any direction on the host (*A. mellifera scutellata*) (Video S1). In general, the legs were located underneath the thorax and the tarsomeres were aligned horizontally to the substrate surface.

Our video recordings show that the claws of the bee lice are primarily responsible for the attachment to the host. The entire honey bee and in particular its thorax is densely covered with setae (hairs). During attachment of the bee lice to the host, the claws were retracted in proximal direction thereby actively pulling through the hairs (Video S2). As a result, the hairs were interlocked in the clamping regions of the claw, which facilitated strong attachment. Remarkably, the attachment force of one bee louse was sufficient enough, to lift up an entire honey bee (Video S3). During contact with the host, the tarsomeres of bee lice showed a strong resilience against lateral displacement and were more prone to torsion. During detachment, the claws were moved in apical direction which led to the release of the hairs from the clamping regions (Video S4). However, most of the time bee lice stayed in a resting position on the thorax of the host. It was



**FIGURE 4** Attachment posture of *Braula coeca* on its host *Apis mellifera scutellata*. (a) Light microscopy image. (b–d) Scanning electron microscopic images of the configuration of claws, pulvilli and tarsomeres during attachment. (e) Interaction between claw and bee hairs (bh). Scale bars: 500  $\mu\text{m}$  (a), 200  $\mu\text{m}$  (b), 50  $\mu\text{m}$  (c, d), 20  $\mu\text{m}$  (e)





**FIGURE 5** Confocal laser scanning microscopy maximum intensity projections showing the material distribution for a metathoracic leg of *Braula coeca*. (a) Ventral view. (b) Ventrolateral view. Scale bars: 50  $\mu\text{m}$ . cl, claw; gh, grooming hairs; pv, pulvillus; t, claw tooth; TA, tarsomeres

observed that bee lice often moved the legs back and forth, to capture bee hairs in close proximity with their claws (Video S2).

While the claws mainly contributed to attachment on hairy surfaces, they showed no big effect during attachment on smooth glass surfaces. During walking or resting on glass surfaces, the claws were bent inwards. This bending did enable proper contact formation of the pulvilli with the substrate (Video S5). The pulvilli enabled adhesion and movement on a smooth surface and adhered strong enough, to avoid detachment, when walking upside down (Video S6). Although the bee lice were able to support their own body weight, the attachment on smooth surfaces was qualitatively much lower than on hairy surfaces. Bee lice easily detached from glass surfaces by pushing them, whereas detachment from a honey bee proved to be much more difficult (Video S7).

Alterations in the behaviour of bee lice were observed during walking on glass surfaces. Bee lice lifted single legs more often from the surface and rubbed them against tarsomere 1 of the corresponding hind leg in an alternating manner (Video S8). The hind legs showed spine-like cuticle outgrowths in this area (Figure 5). This type of behaviour was also observed on hairy surfaces, but less frequently.

### Force measurements of *B. coeca* on honey bees

Force measurement with adult bee lice were performed on freshly killed bees. After proper contact formation, the honey bees were actively pulled off from the bee lice. Figure 3a shows the boxplot of the maximum attachment forces observed for all tested bee lice ( $n = 18$ ). The maximum attachment force was  $16.06 \pm 3.41$  mN and

the corresponding safety factor was  $1130 \pm 300$  (median  $\pm$  mean deviation from the median). The safety factor represents how often the animal can attach its own weight to a substrate (attachment force divided by weight force).

## DISCUSSION

### Attachment on bees and the role of specialized claws

The bee louse *B. coeca* displays exceptional attachment strength on bees and is able to run swiftly in any direction (Video S1). Compared to other common terrestrial ectoparasitic hosts, like birds, reptiles or mammals, bees are rather small hosts and covered with microstructured, tiny setae/hairs (Thorp, 1979), making it difficult to assure reliable, repeatable interlocking and releasing. Bee hairs are highly diverse morphologically, differ in their density in different regions of the bee (Thorp, 1979) and are often contaminated with pollen. In addition, *B. coeca* possibly must also avoid detachment during grooming of the bees (Martin & Bayfield, 2014) and must be able to walk efficiently within the beehive dominated by smooth, wax- and propolis-covered hydrophobic surfaces (Hepburn et al., 2014), where their reproduction and foraging take place (Örösi-Pál, 1938).

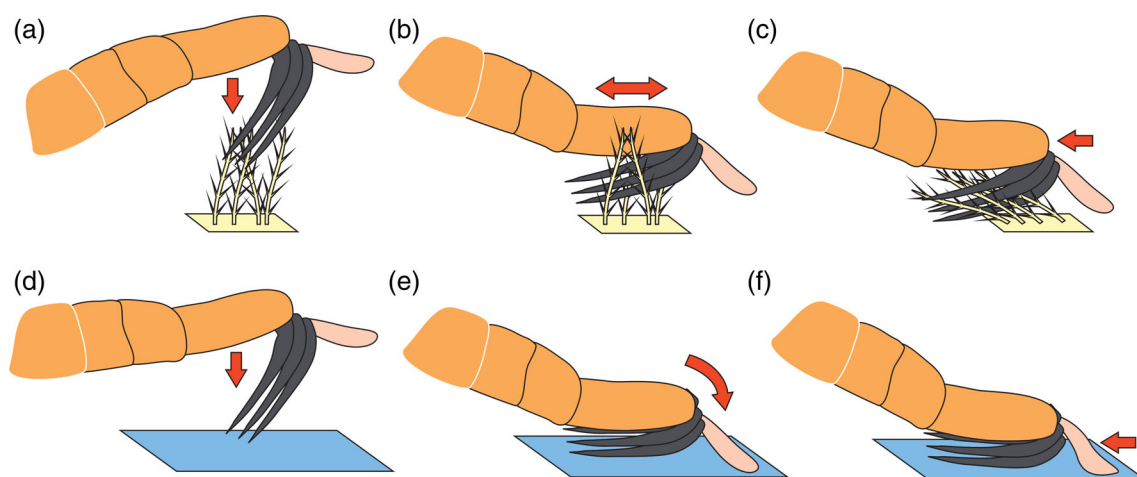
Based on our observations, the claws are the main device for reliable and strong attachment on bees (Figure 4). Typically, representatives of Brachycera, such as the well-known model organism *Drosophila melanogaster* (Meigen, 1830), a close relative of *B. coeca* (Wiegmann et al., 2011), only possess nonserrate, single-tipped claws. In contrast, bee lice have serrate claws with around 15 single teeth per leg. A single tooth has an angle of curvature of  $65^\circ$  (base) to  $16^\circ$

(tip) and the tip-to-tip distance between individual teeth is broader in their distal regions and becomes narrower closer to their bases. Previously, the attachment system of the flightless ectoparasitic louse fly *C. pallida* (Hippoboscidae) was investigated (Petersen et al., 2018). It was shown that they have highly modified, tridentate claws. The claws are used to strongly attach to the feathers of the bird host *A. apus*. They actively pierce the feather vanes, so that single barbs get stuck between the clamping regions. Due to the barb sliding from the wider to narrower gaps between claw teeth, the normal load on the barbs rises and the friction between the claw teeth and barbs strongly increases. External pulling force can even enhance the attachment strength, because friction will even more increase due to the further slipping of the feather structures into the tapered space. A friction system, based on such an interlocking, is very energy efficient and the resulting attachment strength is not limited by the musculature strength of the fly. It is very common among insects and can be found for example in the wing locking devices of beetles or the head arresting system of dragonflies (S. N. Gorb, 2001).

The same principle is used in the attachment system of *B. coeca*. However, overall *C. pallida* has only 24 clamping regions on all legs combined, that is, two interstices per claw that allow for clamping of fibrillar structures. In comparison, *B. coeca* has 28 clamping regions in average on one leg. The teeth of each claw leave 28 interstices in average per foot and hence allow for clamping bee hair in these clamping regions. The reason for this strong increase in the number of clamping regions may be associated with the host, on which the bee lice have to adhere to. Although bees are strongly covered with hairs, the density of microstructures is much lower than on the plumage of birds (Matloff et al., 2020; Thorp, 1979). Furthermore, single hairs of bees are much bigger in relation to the body size of *B. coeca*, if compared to the feather microstructures in relation to the body size of *C. pallida* (Figure 6). Additionally, the interstices between single teeth

of the claw directly correspond to the thickness of the majority of the bee hairs (Figures 4 and 6). The tip-to-tip distance between the claw teeth most likely enables the claw to collect single hairs during movement and then the hairs slide towards the narrowing gaps at the claw base. As the amount of hairs collected in the described manner affects the efficiency of attachment, and the parasites cannot influence the integrity of the collected substrate, some of the measured individuals potentially grasped a higher amount of hairs by chance. This can explain the higher values in the skewed distribution of the attachment forces and safety factors.

*B. coeca* also shows some behavioural adaptations with regard to the interlocking process. Bee lice actively collect bunches of hairs for secure attachment (Video S2, Figure 6b) by a back and forth shearing motion of the legs. The orientation of the claw tips towards the centre of the animal supports the collecting ability. During shearing, the tarsus is also pulled towards the body (Figure 6c). Often bee hairs are serrate and the small distally oriented microstructures could negatively influence the interlocking process by reducing the sliding of the hairs into the clamping region between the teeth of claws. This can be the case, if the serration of the bee hairs avoids proper clamping in the first place. If the hairs are collected, supported by the described shearing motion, the microstructures of the bee hairs can also potentially result in an increased friction. Judging on the relative stiffness of the claws and setae of *B. coeca* (this study) and other insects (Eshghi et al., 2018; Peisker et al., 2013; Petersen et al., 2018; Reborá et al., 2018), the bee hairs are probably less stiff than the claws of the bee louse and therefore clamping of the hairs in the claw interstices, presumably, results in high friction. However, the contact mechanical interaction between single hairs and claws could be subject to subsequent studies, which can also investigate the influence of the different morphologies of the bee hairs from different body regions, sexes or species (Matloff et al., 2020, Thorp, 1979). The shearing motion of the



**FIGURE 6** Scheme showing the attachment process of *Braula coeca* on bee hairs (a–c) and smooth substrates (d–f). (a) First, the claw (dark grey) is open and the tarsus (orange) is pushed towards the base of the bee hairs (yellow). (b) The tarsus then moves back and forth in a shearing motion and the claw tips are oriented towards the Centre of the fly. (c) As a result, the interstices of the claw teeth interlock with the bee hairs. All bee hairs become well aligned to the tarsus and are packed one above another in the interstices. The red arrows correspond to the movement of the entire tarsus. (d) On smooth substrates, the claws do not interlock with hairs. (e) Instead, the claws are folded inwards. (f) The protruding pulvilli (pink) are then brought into contact for attachment



legs can then directly help during the interlocking process with serrate hairs. Once firmly attached, bee hairs are properly ordered on top of another and become well aligned with the tarsomeres of the bee louse (Figures 4a,b and 6c). This shearing motion is controlled by the musculature present in the thickened femur. The main flexor muscle of the tibia is located in the ventral part of the femur (Snodgrass, 1935). Potentially, the enlarged musculature in the femora helps to keep the legs clamped below the body of the bee louse and provides the back and forth drag necessary to interlock with the bee hairs. This requirement to move the legs back and forth may also explain, why the legs are located underneath the thorax, so that only the tarsi are aligned parallel to the bee surface in resting position (Figure 4a). Thorax and abdomen of *B. coeca* are always elevated above the substrate surface which is very untypical for ectoparasites. Normally, the thorax is dorso-ventrally flattened and the entire body is kept very close to the surface to reduce drag and to avoid physical removal by the host (Clayton et al., 2010; Goodman et al., 2020), as it is the case for example in the feather lice *Columbicola columbae* (Linnaeus, 1758) and *Campanulotes compar* (Burmeister, 1838) (both Insecta: Phthiraptera).

In contrast to representatives of several other insect orders (Beutel & Gorb, 2001, 2006, 2008; Büscher et al., 2018, 2019; Friedemann, Spangenberg, et al. 2014; Haas & Gorb, 2004; Nelson, 2009), but typical for dipterans (Friedemann, Schneeberg, et al. 2014), attachment structures on *B. coeca* are exclusively found on the pretarsus. The tarsomeres themselves, consequently, do not bear specialized attachment devices. However, the apical pretarsal attachment devices need to be kept aligned with the tarsus. To avoid lateral deflection of the tarsus and enable stabilization of the tarsal chain in lateral direction, the apical expansions of the tarsomeres may serve as stoppers. For detachment, the mechanical interlocking of the claws with the hairs needs to be separated. This separation may be achieved by a rotational movement of the claws, supported by rapid movements of the leg and the relaxation of the claw retractor muscle (Video S4). The soft lateral ridges (Figure 5b) on the tarsi potentially provide flexibility, to enable contact separation between the claws and the substrate with a rotational (twisting) movement, previously described for the tarsi of houseflies (Niederegger & Gorb, 2003).

CLSM was performed to analyse the cuticle composition of the attachment system of *B. coeca* and to indirectly estimate its material properties. Normally insects use claws for mechanical interlocking with rough substrates, always with the claw tip interlocking with irregularities of the substrate (Bullock & Federle, 2011; Büscher & Gorb, 2019; Bußhardt et al., 2014; Dai et al., 2002; Patrick et al., 2018; Song et al., 2016). The claw tips of these insects often are reinforced and show high material strength (Dai et al., 2002; Fontaine et al., 1991). To our surprise, the tips of single claw teeth in *B. coeca* appear to be softer than the remaining claw parts, similar to the case observed in the *Ixodes ricinus* (Linnaeus, 1758) ticks (Voigt & Gorb, 2017). However, it is important to keep in mind that in *B. coeca* the function of the claw tips is fundamentally different from most other insects. Unlike in other insects, the tips are not exposed to stress concentration during mechanical interlocking. We believe, that the soft tips could be important during the attachment process on bee hairs. The deformation of

the claw tips may help to collect bee hairs more efficiently by providing additional guidance for the bee hairs into the interstices. Also, the hook mobility may provide more uniform attachment strength in different directions, as it has previously demonstrated in plant burrs (E. V. Gorb et al., 2002). The major stress distribution in *B. coeca* claws is limited to the narrow regions of the tooth base, in which the CLSM analysis also indicates a higher sclerotization than at the tip.

## Attachment on smooth substrates and the role of pulvilli

The bee louse *B. coeca* does not only attach to bee hairs, but also has to attach to smooth surfaces like the waxy surfaces found in the beehive (Hepburn et al., 2014). The special arrangement of claws and pulvilli favours a division of labour between mechanical interlocking with hairs on the bee (claws), and attachment on other surfaces including rather smooth ones (pulvilli). Bee lice can ‘walk’ on their claws (Video S5), if they are completely folded inwards (Figure 6d-f). In doing so, the pulvilli are able to get in proper contact with the corresponding surface. The material composition of the pulvilli is dominated by resilin, which indicates soft mechanical properties (Eshghi et al., 2018; Peisker et al., 2013; Petersen et al., 2018). The ventral face of the pulvilli is covered with setae known to be essential to generate adhesion on smooth surfaces due to capillary forces and van der Waals forces (Langer et al., 2004). Interestingly, each pulvillus has approximately 120–150 setae, while the closely related fruit fly *D. melanogaster* shows only about 30 setae per pulvillus (Hüsken et al., 2015). An increase in setal density leads to a higher real contact area with the substrate and higher attachment strength, given that the spatulate tips of the setae have similar dimensions. The dimension and shape of the spatulas of the wild-type *D. melanogaster* is approximately the same compared to *B. coeca* (mean width 1.97 µm according to Hüsken et al., 2015). In summary, various morphological adaptations of bee lice may suggest that its attachment to smooth surfaces is similarly important as to the bee hairs.

## Exceptional safety factors of bee lice

In this study, the morphological and behavioural adaptations of *B. coeca* are described. In addition, force measurements on bees were performed, to quantitatively evaluate the attachment ability (Figure 3a). *B. coeca* showed exceptionally high attachment forces on bees with a safety factor higher than 1000. To put the attachment ability of *B. coeca* into general context of insect adhesion, safety factors for different terrestrial insect groups were collected from the literature (Figure 7, see Table S3 for a detailed list of all sources). We always considered the highest safety factor for a species mentioned in the studies to perform a more conservative evaluation. It is important to mention that safety factors from the literature were obtained on various surfaces and with different experimental methods, so that a direct comparison with the values obtained in the present study is limited, but only a coarse comparison is well possible. Apart from ants (Formicidae), the swift louse *C. pallida*, a highly

specialized avian ectoparasite, and *B. coeca*, no other terrestrial insect group generated safety factors higher than 100. Only both dipteran ectoparasites reach safety factors of around 1000. To our knowledge, *B. coeca*'s safety factor of 1100 in median is the highest one ever measured for terrestrial insects. Other nonparasitic dipterans normally show safety factors between 25 and 35.

Higher safety factors are easier to reach, if an organism is smaller due to their higher volume-to-area ratio and easier uniform load distribution onto the contact area (S. N. Gorb et al., 2001; Labonte & Federle, 2015). However, other insect groups, comparable in size to *B. coeca*, like aphids, do not show these high safety factors, and *C. pallida* also displays exceptional safety factors while being a larger organism with a size of up to 1 cm (Petersen et al., 2018). Figure 7 clearly shows the huge disparity between the attachment ability of ectoparasites and other freely living insects from different systematic groups. It is fair to assume that reliable, strong attachment is one of the most important aspects for biology of ectoparasites having a strong evolutionary pressure for staying on their hosts. This evolutionary pressure explains the extraordinary modifications of the parasite attachment system. In the case of *B. coeca*, it uses a temporary attachment system that enables easy attachment/detachment to/from the surface and fast movement on the host. Other organisms that reach such high safety factors normally rely on glues and attach permanently to a surface (e.g., barnacles, tubeworms or mussels: Oliveira & Granhag, 2016; or insect eggs: Büscher, Quigley & Gorb, 2020; Büscher, Lohar, et al. 2020). The high attachment forces reached by the bee lice are very likely a result of the host specialization. While the bee lice are able to run freely on a nonflying bee, the most difficult situation for attachment is most likely during flight of the bee, which involves considerable complex flight manoeuvres and drag forces resulting from the air flow (Khurana & Sane, 2016; Luu et al., 2011), at comparably high flight speeds around  $7.5 \text{ m s}^{-1}$  (Wenner, 1962). As the forces experienced by the bee louse depend on several factors, such as the thickness of the

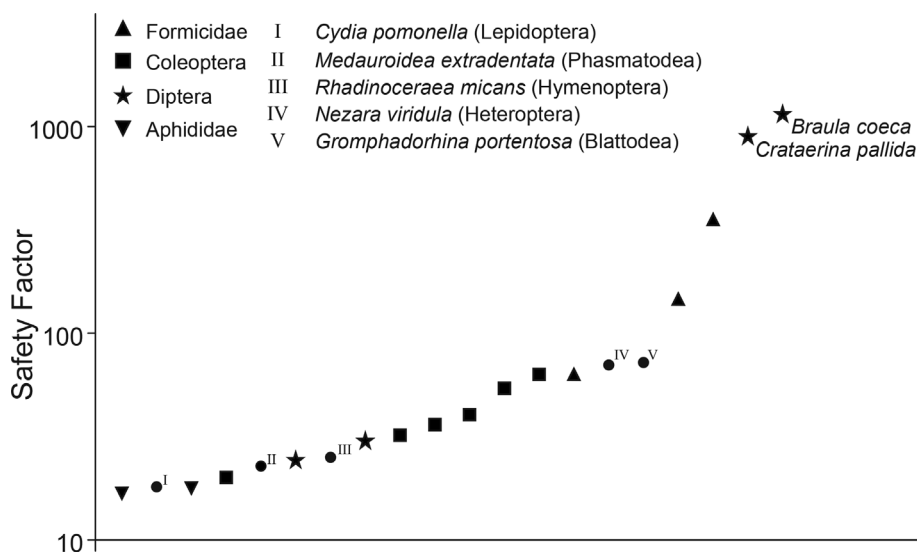
boundary layer and the distribution of airflow fields on the surface of the bee, the pressure distribution, the body angle of the bee (Nachtigall & Hanauer-Thieser, 1992), as well as on the morphological features at the position of attachment, these forces can be very different. Therefore, to sustain proper attachment to the bee, such high safety factors can be necessary. For an estimation of the forces experienced by the bee lice during flight of a bee, we calculated the drag force a single bee louse is subjected to on the most exposed area of the bee. We used the following formula:

$$D = \frac{C_d \rho S v^2}{2}, \quad (1)$$

where  $C_d$  is the drag coefficient,  $S$  is the area resisting the air flow and assuming a flight speed ( $v$ ) of  $7.5 \text{ m s}^{-1}$  (Wenner, 1962), an air density ( $\rho$ ) of  $1.3 \text{ kg m}^{-3}$  and the drag coefficient of a sphere (Table S2 for all input values and the calculation). The resulting drag force (0.027 mN) at full exposure is much lower than the average attachment force of the bee lice. The attachment force of *B. coeca* is approx. 590 times (median attachment force divided by the calculated drag force) stronger than the experienced drag force on the most exposed place on the bee. Consequently, although this estimation is somewhat simplified, it can be assumed that the bee lice find sufficient attachment on the bee during flight. Furthermore, the bee lice can also find shelter in regions on their hosts' bodies, which are less exposed, to avoid unfavourable conditions on the bees' surface.

### Cleaning and maintenance of the attachment system

As corroborated from our observations (Video S8), *B. coeca* cleans the claws of all legs, using the grooming setae of the metathoracic legs. The bee lice bring the tarsi of front and mid legs respectively together with



**FIGURE 7** Safety factors of *Braula coeca* and other insects reported in the literature. y-Axis has a logarithmic scale. A detailed list of all publications and reported safety factors can be found in Table S3

the grooming patches of the hind leg and actively scratch the hind tarsus over the claws. Hind leg tarsi are cleaned with the contralateral hind tarsus as well. With this behaviour the claws are cleaned from residues remained in the claw interstices. The intimate contact between the louse claws and bee setae potentially may result in ripping off single bee setae. These setae can potentially contaminate the louse claw interstices and, hence, hinder proper attachment to the host. In contrast to the claw tips, the grooming setae appear homogeneously stiff according to their green autofluorescence and even stiffer than the tips of the corresponding claws, which reveal a gradient in their material distribution. As the grooming setae have similar or smaller diameter than bee hairs (~3.4–5.3 µm)—and an even thinner tip—they are able to fit into the interstices between the teeth, to mechanically remove bee hair residuals from the claws. While doing so, the setae are probably used, to comb bee hairs out of the claw comb. Grooming devices in other insects, for example, bees or true bugs, are often used for cleaning from pollen or other smaller particles, and, hence, are more lamellate in shape (Basibuyuk & Quicke, 1995; Schönitzer, 1986; Schönitzer & Lawitzky, 1987; Schönitzer & Renner, 1984) and reveal a stronger material gradient getting softer towards the tips (Rebora et al., 2019). However, these grooming devices are used to scratch off fine particles and rub the collected contaminations from the cleaning devices. In contrast, the cleaning device of *B. coeca* probably scratches larger pieces of cuticular materials, that is, ripped off bee hairs, out of the interstices of the claws.

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#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHOR CONTRIBUTIONS

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N. Bijma, Fabian Bäumlner and Sebastian Büsse. **Supervision:** Stanislav N. Gorb. **Project administration:** Dennis S. Petersen and Thies H. Büscher.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study, Tables S1 and S3, as well as Videos S1–S8, are openly available in figshare at <https://doi.org/10.6084/m9.figshare.15062694>.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

**Table S1.** Dataset of the measured maximum attachment forces, masses and safety factors of the tested individuals.

**Table S2.** Table of the parameter for the estimation of the drag force a single *B. coeca* is exposed on the surface of a bee.

**Table S3.** Table with all publications used for the creation of Figure 7. Additional information is given with respect to evaluation method and substrate.

**Video S1.** Supplemental movie showing the movement of *B. coeca* individuals on anaesthetized bees.

**Video S2.** Supplemental movie showing the leg movements of *B. coeca* for collecting bee hairs with the claws

**Video S3.** Supplemental movie showing the strength of attachment of *B. coeca* to a bee by lifting the bee with a bee louse attached to a needle.

**Video S4.** Supplemental movie showing the release of the collected hairs.

**Video S5.** Supplemental movie showing the locomotion of *B. coeca* on a smooth substrate.

**Video S6.** Supplemental movie showing the attachment of *B. coeca* to a glass slide from ventral view.

**Video S7.** Supplemental movie showing the different attachment performance of *B. coeca* individuals on smooth substrates in comparison to individuals attached to the surface of a bee.

**Video S8.** Supplemental movie showing the cleaning motion of *B. coeca* individuals using their hind legs.

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