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Article:

1   **Constraints on Convergence: Hydrophobic Hind Legs Allow Some Male Pollinator**

2   **Fig Wasps Early Access to Submerged Females**

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1   Abstract

2   Pollinator fig wasps (Hymenoptera: Agaonidae) display numerous adaptations linked

3   to their obligate association with fig trees (*Ficus*). *Ceratosolen* fig wasps pollinate figs

4   that often fill temporarily with liquid and one clade has males with unusually long hind

5   legs. We investigated their morphology and behaviour. SEMs revealed that the cuticle

6   of their hind legs is highly modified and covered with numerous hydrophobic setae and

7   microtrichia that can prevent blockage of the wasps' large propodeal spiracles by

8   liquids. In deep liquid, the males floated on the surface, but when only a thin layer of

9   liquid was present, the legs allowed males to access females without the risk of

10   drowning. Access to females was facilitated by an air bubble that forms between the

11   hind legs and maintains a column of air between the spiracles and the centre of the figs.

12   Sexual selection should favour males that can gain earlier access to mates, and the

13   modified legs represent an adaptation to achieve this. Convergent adaptations are

14   known in some unrelated non-pollinating fig wasps that develop in similar liquid-filled

15   figs, but these species have enlarged hydrophobic peritremata at the ends of their

16   metasoma to protect the spiracles located there. Unlike non-pollinating fig wasps,

17   pollinator males need to insert their metasoma deep into females' galls during mating.

18   This difference in mating behaviour has constrained the extent of convergence.

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22   Key words: Agaonidae; aquatic insects; drowning; *Ficus*; respiratory adaptations;

23   sexual selection

24

1   **Introduction**

2   Water bodies exist at a wide variety of spatial scales, from open oceans and large lakes to

3   roadside puddles and individual rain drops deposited onto plants or the ground. For small

4   insects, water at all these scales is potentially hazardous, and has resulted in hydrophobic

5   (water-repellent) properties being a general feature of the bodies of terrestrial insects (Byun

6   et al. 2009; Hu et al. 2011; Pedersen 2012). This ability to repel water is usually attributed

7   to physical features, such as hair piles, rather than chemical properties of the cuticle

8   (Holdgate 1955; Balmert et al. 2011; Hu et al. 2011). Aquatic insects differ in that their

9   cuticles are usually hydrophilic (Holdgate 1955), but they also often have hydrophobic

10   areas associated with their spiracles (Chapman et al. 2012).

11

12   Water is not the only liquid encountered by insects. Liquid exudates from plants such as

13   resins can trap insects and latex is an effective physical and chemical defence against non-

14   adapted herbivores (Compton 1987; Stankiewicz 1998). Plant exudates can also modify

15   the properties of the liquids held in phytotelmata – modified plant structures forming

16   rainwater-trapping containers that provide protection from herbivores or act as traps from

17   which the plants can extract nutrients (Kitching 2000). Plants also routinely secrete floral

18   exudates (nectar) to reward pollinators and exceptionally large quantities of exudates are

19   involved in the pollination of some orchids, where the pollinator bees are required to crawl

20   through the liquid to acquire pollinia (Dodson and Frymire 1961). Exudates from

21   inflorescences can also have a defensive function (Monteiro and Macedo 2014) and some

22   fig trees (Moraceae: *Ficus*) produce exudates that temporarily fill the central cavity of their

23   highly specialised hollow inflorescences (figs, sometimes called syconia). The function of

4

1 the liquid is unknown (Compton and McLaren 1989), but Janzen (1979) believed it has

2 antimicrobial properties.

3

4 Fig trees are a large group of mainly tropical plants that are pollinated by fig wasps,

5 (Hymenoptera: Chalcidoidea: Agaonidae), and fig wasps can only reproduce within their

6 figs. They are potentially exposed to any liquid in the figs, as are the numerous species of

7 non-pollinating fig wasps (NPFW, also Chalcidoidea) that exploit the mutualism. Fig trees

8 and fig wasps have a long history of association (Compton et al. 2010; Cruaud et al. 2012;

9 Farache et al. *in press*). Each fig tree species is pollinated by one or a small number of fig

10 wasps, most of which are host tree specific. In order to lay their eggs, adult female

11 pollinators enter receptive (B-phase *sensu* Galil and Eisikowitch 1968a) figs through a

12 narrow entrance called the ostiole. Once inside, they lay eggs into the numerous ovules

13 that line the inner wall of each fig, gall the ovules and also actively or passively pollinate

14 them (Janzen 1979; Weiblen 2002). One larva develops inside each ovule and the next

15 generation of female pollinators mate, vacate their galls and acquire pollen before leaving

16 the figs via holes chewed by the males (Galil and Neeman 1977; Weiblen 2002; Suleman

17 et al. 2012). Most NPFW have females with long ovipositors allowing them to lay their

18 eggs from the outside of the figs, and so have no opportunity to transfer pollen. Their

19 biology is diverse, but most either independently gall the ovules, or develop at the expense

20 of pollinators or other gall-formers (Chen et al. 2013).

21

1 Adult male pollinators are wingless and spend all or most of their lives inside their natal

2 figs. As in many insects, they emerge from their galls before the females. They seek out

3 galled ovules containing conspecific females and chew holes through the galls before

4 inserting their telescopic metasoma to achieve mating. NPFW males emerge at about the

5 same time, and mating commonly takes place in the lumen of the figs or outside the fig,

6 depending on the species.

7

8 The male fig wasps associated with fig tree species belonging to *Ficus* Subgenus

9 *Sycomorus* routinely emerge from their galls into a fig lumen that is still partially filled

10 with liquid. Males of some of the NPFW associated with these figs possess highly modified

11 peritremata that surround the spiracles located towards the end of their metasoma and can

12 extend into whip-like appendages (Galil and Eisikowitch 1968b). The peritremata have

13 water-repellent properties and allow the insects to search for mates under conditions that

14 would otherwise pose a risk from drowning (Compton and McLaren 1989; Ramirez 1996).

15 Male pollinator fig wasps do not possess similarly-enlarged peritremata at the end of their

16 metasoma, but males of some of the *Ceratosolen* species that pollinate *Sycomorus* figs have

17 modified hind legs that might serve a similar function (Wiebes 1994, Figure 1). These

18 species were distinguished as *Ceratosolen* Subgenus *Rothropus* by Wiebes (1994).

19

20 The modified legs of these species may allow them to search earlier for females in what is

21 a potentially hazardous environment. Here, we first describe the morphology of the

22 modified hind legs of one *Ceratosolen* species and contrast them with those of a congener

6

1   with unmodified legs. We then present the results of experiments to compare the water-

2   repellent properties of their legs and describe the behaviour of the insects in relation to the

3   amounts of liquid present in the figs, to ascertain how males use their legs when searching

4   for mates. The results provide insights into how convergent adaptations to adverse

5   environments among insects can arise as a result of sexual selection, but also that the extent

6   of convergence can be limited by basic aspects of the insects' anatomy and behaviour.

7

8   **Materials and Methods**

9   **Study species and sample collections**

10   We studied males of two pollinator fig wasp species associated with Asian fig trees

11   belonging to *Ficus* subgenus *Sycomorus.* These trees are gynodioecious, and the fig wasp

12   offspring only develop in figs on male plants. *Ceratosolen corneri* Wiebes is the pollinator

13   of two closely-related fig tree species, *F. botryocarpa* Miq. and *F. linearifolia* Elmer, and

14   has males with modified long hind legs. Based on *COI, cytochrome b* and *elongation factor*

15   *1α* gene trees, *C. corneri* belongs to a clade of pollinator wasps that all possess modified

16   hairy legs and *Ceratosolen bisulcatus* (Mayr) belongs to a sister group of this clade (Moe

17   and Weiblen 2010; Cruaud et al. 2012). *Ceratosolen bisulcatus* pollinates *F. septica*

18   Burm.f.. Its males have unmodified hind legs that are typical of the remainder of the genus,

19   and male pollinator fig wasps in general. Figs of *F. botryocarpa* and *F. linearifolia* and

20   their associated fig wasps were collected from Mount Makiling Natural Park, Los Baños,

21   Laguna, Philippines (14.14°N 121.23°E). *Ficus septica* figs were collected from the

22   University of the Philippines Diliman campus, Quezon City, Metro Manila, Philippines

23   (14.66°N 121.07°E). Sampling was performed from May 2015 to January 2016.

1

Male fig wasp morphology

We used scanning electron microscopy (SEM) to compare the morphology of the hind legs of the two *Ceratosolen* species. Male wasps of *C. corneri* were collected only from *F. botryocarpa*. The wasps were air-dried and coated with a gold-palladium coating using Hitachi E-1010 Ion Sputter (Japan). They were examined using a Hitachi S3400N scanning electron microscope (Japan). The software ImageJ v.1.47 was used to record quantitative features such as setae lengths, distances between setae and microtrichia lengths. Our terminology is modified from Balmert et al. (2011) and Matheson (2013), where setae and trichoid sensilla are described as having a socket or annular base, whereas microtrichia are non-cellular processes of the epicuticle, with no socket or base.

Fig liquid characteristics and male emergence

C-phase and D-phase (*sensu* Galil and Eisikowitch 1968a) male figs of the three fig species were collected and cut in half through the ostiole and peduncle. (n = 30-31 figs per species). These developmental phases span the period from when fig wasp larvae are developing through to when adult female wasps emerge from the figs. The amount of liquid found inside the cavity of each fig was rated using a visual scale - 0: No liquid visible within the central cavity, 1: Only ovules and seeds covered by liquid, 2: Flowers entirely covered by liquid, 3: Central cavity up to 50% full of liquid, 4: Central cavity >50% full of liquid. The quantities of liquid were then related to the numbers and behaviour of male wasps that had emerged from their galls into the central cavity.

1

2   We estimated the surface tension of the liquid inside male figs of *F. botryocarpa* using a

3   simplified capillary rise technique. The rise of a liquid up a capillary tube is related to its

4   surface tension (Alexander and Hayter 1971). Fig liquid samples were taken from early C-

5   phase, late C-phase and early D-phase figs and the distances they rose up vertically-held

6   capillary tubes was compared with movements of methanol solution standards and distilled

7   water. This was repeated three times for each liquid treatment. Eight methanol dilutions

8   were used to provide liquids of varying surface tension (30, 40, 50, 60, 70, 80, 90 and

9   100%), (Compton and McLaren 1989). The surface tension of the dilutions were then

10 calculated at 25°C using the method of Vazquez et al. (1995) (Table S1).

11

12 **Liquid-repellent properties of male hind legs**

13 We compared the water-repellent abilities of the hind legs of male *C. corneri* from *F.*

14 *botryocarpa* and *C. bisulcatus* after they were submerged in liquids with a range of

15 different surface tensions. Recently-emerged live males were first fixed ventral side up

16 onto petri plates by wedging them using size zero entomological pins. This was necessary

17 to prevent the wasps from floating to the surface of the test liquids. The wasps were then

18 submerged in one of the 10 liquids (eight methanol concentrations, distilled water and early

19 C-phase fig liquid). Each fig wasp was used only once, with 10 fig wasp replicates per

20 liquid. The extent of air bubble formation around the hind legs was then scored accordingly

21 - 0: No air bubble, with full wetting, 1: Small air bubble present, with partial wetting of the

9

1   hind legs or propodeal spiracles, 2: Air bubble formed along the length of the hind legs,

2   covering the propodeum, with no wetting of the hind legs.

3

4   **Behavioural observations of *Ceratosolen* males**

5   Figs containing varying amounts of liquid were cut into two halves as before. The

6   behaviour of the male fig wasps was then observed for the next 10 minutes through a

7   stereomicroscope and related to the amounts of liquid present in the figs.

8

9   **Data analysis**

10   All analyses were performed in R version 3.2.1 (R Development Core Team, 2015).

11   Quantitative measurements of the pilosity of *C. corneri* and *C. bisulcatus* legs were

12   assessed mainly using One-way ANOVA followed by Tukey's HSD tests. A generalised

13   linear model (GLM) with quasi-Poisson errors compared setal distances of *C. bisulcatus*,

14   because of over-dispersion and non-homogeneity of errors. GLMs with quasi-Poisson

15   errors were also used to compare male fig wasp activity inside figs containing different

16   quantities of liquid, again due to over-dispersion of count data. Similarly, the relationship

17   between liquid surface tension and the ability of the fig wasps to form air bubbles around

18   their legs was assessed using One-Way ANOVA for *C. corneri* and a GLM with quasi-

19   Poisson errors was used for *C. bisulcatus*.

20

21   **Results**

1 **Male fig wasp morphology**

2 Adult males of *C. bisulcatus* were yellowish-brown, with a body length of about 1 mm

3 (Figure 2). The hind legs were relatively stout and had a length of about 0.7 mm, with

4 coxae, femora and tarsi of about the same length, and shorter tibiae. The segments had a

5 simple, uniform cuticle and were sparsely and irregularly covered with a grooved surface

6 and socketed trichoid setae layer that is likely to have a mechanoreceptive tactile function

7 (trichoform sensilla) (Li et al. 2009; Matheson 2013; Figure 2). Setae located within

8 sockets were present on all the segments of the hind legs of *C. bisulcatus*, other than the

9 coxa. The second tarsal segment had the most setae per unit area and the first tarsal segment

10 had the least (Table 1). Setae from the first and second tarsal segments had the longest

11 mean lengths, and those on the tibia and femur had the shortest (One-way ANOVA:

12 $F_{5,14}$=11.55, P<0.001, Table 2). The setae had grooved surfaces and were bent over at the

13 tips (Figure 3). Only setae with sockets were recorded.

14 As expected, there were no differences noted in any of the features of the hind legs of *C.*

15 *corneri* reared from *F. botryocarpa* and *F. linearifolia*. Adult males of *C. corneri* were

16 light brown, with a body length of about 2 mm. In comparison with *C. bisulcatus*, all the

17 segments of their hind legs were elongate, resulting in a total length that was about equal

18 to that of the body (Figure 4). The tarsi also formed an arc that curved forward from the

19 tibia, rather than continuing in the same plane as the femur and tibia (Figure 5). The joint

20 between the femur and tibia was unusually articulated and allows the legs to bend sideways,

21 away from the main axis of the body.

22

11

1   In contrast to *C. bisulcatus*, the cuticle on the inner proximal surface of the hind coxae of

2   *C. corneri* was covered in a dense mat of irregular protrusions (Figure 6). Elsewhere, on

3   the inner surfaces of the femur and tibia, and the entire surface of the tarsi except close to

4   the claws (and wherever dense long setae were present), this was replaced by a covering of

5   short, spine-like microtrichia. These extended onto the bases of some of the prominent

6   setae, if they lacked sockets (Figure 7). The densities of microtrichia varied within

7   individual segments. They averaged about 0.8 µm in length, which was roughly 5% of the

8   length of the setae. The length of the microtrichia varied slightly between segments, with

9   significant differences in mean length (One-way ANOVA: $F_{7,152}$=3.01, P<0.01, Table 2).

10

11   The setae extended well beyond the microtrichia. They were elongate and tapering, and

12   had longitudinally grooved surfaces (Figures 6 and 7). Many of the setae were bent over

13   towards their tips. This varied in extent, but the bend was often abrupt (Figure 7). The setae

14   situated on the inner face of the coxae were different from those present on other segments,

15   with bases located in raised, cup-like extensions of the cuticle (Figure 6). Elsewhere, a

16   minority of the setae emerged from basal sockets and were similar in appearance to those

17   present in small numbers on the legs of *C. bisulcatus*. Setae with sockets were most dense

18   on the second tarsal segment. Microtrichia did not extend onto the bases of these socketed

19   setae. In addition to the areas with large numbers of socket-free setae, small numbers of

20   socketed setae were also scattered on the outer sections of the coxa, femur and tibia, in

21   areas of the legs where setae that lacked sockets were absent. There were no differences in

22   the lengths of the setae with sockets between different segments of the legs (One-way

23   ANOVA: $F_{3,5}$=0.56, P=0.67). Setae with sockets had a mean length of about 26.5 µm,

1 significantly longer than the 18.0 μm mean length for setae without sockets (One-way

2 ANOVA: $F_{1,205}$= 44.51, $P<0.001$).

3

4 The setae without sockets were at much higher densities than those with sockets (Table 1).

5 The socket-free setae on different segments were relatively uniform in length (Table 1) but

6 lengths nonetheless did vary significantly (One-way ANOVA: $F_{7,167}=6.073$, $P<0.001$), as

7 a result of the setae on the fifth tarsal segment being longer than those on the tibia, femur

8 and first tarsal segment (Tukey's HSD: P all <0.01, Table 1). There were also significant

9 differences in the distances separating socket-free setae on different segments (One-way

10 ANOVA: $F_{7,173}=14.44$, $P<0.0001$), with the setae spaced further apart towards the end of

11 the legs (Figure 6, Table S2). This was reflected in a corresponding decline in the density

12 of the socket-free setae towards the end of the legs with significantly more socket-free setae

13 per unit area on the tibial, femoral and coxal segments than the second and third tarsal

14 segments (One-way ANOVA: $F_{7,9}=9.66$, $P<0.01$; Table S3).

15

16 The overall lengths and densities of the setae with sockets did not differ significantly

17 between the two *Ceratosolen* species (Length: One-way ANOVA: $F_{1,27}=0.04$, $P=0.85$;

18 Density: GLM: $F_{1,8}=3.54$, $P=0.10$; Table 1) and their overall appearance was similar.

19

20 **Fig liquid content and male wasp activity**

1   The central cavities of the male figs of *F. botryocarpa*, *F. linearifolia* and *F. septica* were

2   largely filled (more than 75%) with liquid during the period when pollinator larvae were

3   developing inside the galled ovules. This liquid was then reabsorbed by the fig at about the

4   time that the adult male pollinators began to emerge (Table 2). In figs of *F. botryocarpa*

5   and *F. linearifolia,* the *C. corneri* males routinely emerged when liquid was still present

6   around the galls, and some rather small males emerged earlier and were exposed to more

7   liquid. The emergence pattern among *C. bisulcatus* males in *F. septica* figs was different,

8   with few males recorded in figs where the liquid had not been fully reabsorbed.

9

10   **Fig liquid characteristics**

11   The extent of capillary rise is correlated with the surface tension of liquids. Using the

12   methanol concentration standards, the surface tension of the liquid inside the cavities of

13   early C-phase, late C-phase and early D-phase figs of *F. botryocarpa* were estimated as

14    $36.51\text{-}41.09$ Nm^{-1} x 10^7, $29.83\text{-}32.86$ Nm^{-1} x 10^7 and $23.93\text{-}27.48$ Nm^{-1} x 10^7 respectively.

15   The liquid inside the figs had a lower surface tension than that of distilled water. Its surface

16   tension declined as the figs matured and the liquid became less abundant (One-way

17   ANOVA: $F_{11,38}=23.84$, $P<0.001$; Figure 8; Table S4). Due to the fig liquid having a lower

18   surface tension than water, resistance to wetting was always more difficult to achieve in

19   fig liquid than in more typical aquatic habitats. Moreover, resistance to wetting was

20   particularly difficult in the less abundant fig liquid at late C-phase, when *C. corneri* males

21   were emerging from their galls.

22

1   **Resistance to wetting of male hind legs**

2   Air bubble formation provided a visual measure of the resistance to wetting exhibited by

3   the hind legs of the male fig wasps when they were placed in liquids with differing surface

4   tensions. The legs of *C. corneri* were able to resist wetting by liquids with lower surface

5   tensions than any of those recorded for the liquid inside the figs, but resistance to wetting

6   by the hind legs declined in line with reductions in surface tension (GLM: $F_{9,119}=23.02$,

7    $P<0.001$; Figure 9A). In contrast, the hind legs of *C. bisulcatus* rarely displayed any air

8   bubble formation, irrespective of the surface tension of the liquid. Consequently, liquid

9   surface tension had no significant effect on the extent of wetting of its hind legs (GLM:

10   $F_{9,90}=0.003$, $P=1$; Figure 9B).

11

12   *Ceratosolen corneri behaviour*

13   Males that were crawling around in the central cavity of figs used the front four legs to

14   keep hold onto the surface of galls if most or all of the liquid had been withdrawn (liquid

15   ratings 0-2) The hind legs remained immobile and were extended behind the insect during

16   these movements. When the males descended between the galls, the hind tarsal claws were

17   then used to hold on to the surface of more centrally-located galls, providing stability while

18   the males chewed into galls and then mated with the females inside by inserting their

19   metasoma. If some liquid was present (liquid ratings 1-2), this involved the head and thorax

20   becoming partially submerged in the fig liquid, while the hind legs and propodeal spiracles

21   remained clear of the liquid. When the males wished to move elsewhere, bending of their

1 hind legs drew them back from the liquid and they could then proceed to walk over the

2 surface of the galls again.

3 Small numbers of emerged males were present in figs that had 50% or more of their central

4 cavity filled with liquid. They either became fully submerged or floated on the surface of

5 the liquid. Those that were submerged were usually found dead, or quickly died. Those

6 that floated on the surface held their hind legs in a characteristic way (Figure 10) with the

7 flattened and setae-free areas of the femora resting on the surface of the liquid. The hind

8 legs in combination formed a trapezium shape when viewed from behind. Their liquid

9 repellent properties resulted in the surface of the liquid extending downwards between the

10 legs onto the base of the abdomen and the propodeal spiracles located there. This had the

11 appearance of an air bubble trapped between the legs, but it remained continuous with the

12 surface of the liquid. Males in this position were usually immobile, but could remain alive

13 for at least 30 mins and when removed from the fig liquid they became active and behaved

14 normally when placed on the surface of galled ovules. In contrast, males that were fully

15 submerged and completely wet did not return to the head-down floating position and

16 became comatose. Three submerged wasps that remained below the surface of the liquid

17 were observed with isolated air bubbles between the hind legs and abdomen. Although

18 alive, they were incapacitated by the buoyancy of the air bubble and could not control their

19 movements inside the liquid.

20

21 **Discussion**

1   Most of the male *Ceratosolen* fig wasps that develop in *Sycomorus* figs could become

2   exposed to liquid in their natal figs if they emerge too early from their galls, and thereby

3   risk drowning. Consequently, although the function of this liquid is unclear, it has the

4   effect of delaying the time when it is safe for males to emerge and seek out mates. Within

5   the clade of *Ceratosolen* that includes *C. corneri,* an evolutionary response to this

6   constraint on emergence times has been adaptations that prevent wetting of their major

7   spiracles, allowing them to emerge sooner. A molecular phylogenetic analysis that

8   includes the majority of species in the genus suggests that these adaptations arose just

9   once within an ancestral species (J-Y Rasplus, unpublished).

10   *Ceratosolen bisulcatus* provides an example of what is likely to be typical behaviour in

11   *Ceratosolen* species with unmodified hind legs. The adult males of *C. bisulcatus* rarely

12   emerged from their galls in figs of *F. septica* before most or all of the liquid in the central

13   cavity had been withdrawn. Consequently, they largely avoided contact with the liquid and

14   did not expose themselves to the associated risk of drowning. In contrast, the males of *C.*

15   *corneri* emerged at an earlier stage in liquid withdrawal, and without their unusual

16   combination of behavioural and morphological adaptations to preclude drowning would

17   have been routinely at risk of having the liquid block their spiracles. The elongated hind

18   legs of this species act together to form a tunnel of air that extends from the surface of the

19   liquid down to the large propodeal spiracles. The water-repellent properties of the hind legs

20   are the result of long, dense, hydrophobic hairs that surround the more distal tarsal

21   segments and cover the inner face of the more basal segments. The liquid to which they

22   are exposed to in their host figs has a lower surface tension than that of water, but our

23   experiments showed that they are capable of repelling liquids with such low surface

1 tensions. It appears that the water-repellent properties of their hind legs allow *C. corneri*

2 males to maintain aeration of their propodeal spiracles for prolonged periods when they are

3 partially submerged in liquid, as can occur during mating, but do not facilitate survival of

4 individuals that undergo complete submersion.

5

6 In their survey of insects with hydrophobic cuticles, Balmert et al. (2011) recorded

7 examples of species with surfaces supporting either large and sparse setae; small setae at

8 higher densities (microtrichia); or with both types of setae organised into two layers. The

9 hind legs of *C. corneri* provide a further example of the latter. Balmert et al. (2011)

10 hypothesised that such surfaces with two layers might retain a thick layer of air for short

11 periods in combination with a more persistent thin film of air maintained by the

12 microtrichia. They also concluded that the density of the setae was the most important

13 factor determining the persistence of films of air that become trapped. Few fig wasp

14 males are likely to survive much more than 24 hours, so endurance of the air-film is

15 likely to be less significant than an ability to resist fig liquids that vary in physical

16 properties and have a lower surface tension than pure water. The bending of the long

17 setae on the legs of the fig wasps will aid with the required resistance to wetting (Tuteja

18 et al. 2007) and the grooves on the surface of the setae might also aid in resisting wetting

19 (Guo et al. 2015), but the presence of similar grooves on the setae of *C. bisulcatus*

20 suggests that this is not a derived feature. Trichoid sensilla are often chemoreceptors

21 (Nakanishi et al. 2009), but the relatively low-density socketed setae in the two

22 *Ceratosolen* species are likely to share a similar mechanoreceptive function (Richards

23 and Richards 1979; Ravaiano et al. 2014; Zhang et al. 2015). They may be the original

1 form from which the much more abundant socket-free setae on the legs of *C. corneri* are

2 derived. Together with the major increase in their numbers and density, the change in the

3 bases of the setae would then be associated with a change in function, from sensory

4 reception to liquid repellence. The functions of the microtrichia and the highly modified

5 cuticle of the hind coxa remain unknown.

6

7 The setae on the hind legs of *C. corneri* bear a striking resemblance to those on the elytra

8 of the semi-aquatic beetle *Galerucella nymphaea* (Coleoptera: Chrysomelidae) described

9 by Balmert et al. (2011). They share the same shape and grooved surface, and are also bent

10 over, though in the beetle the bending is more regular and directional than that seen on the

11 fig wasp. The setae on the elytra are also approximately the same length as those on the

12 legs of the fig wasp, despite the far larger size of the beetle, but are present at a slightly

13 lower density. The elytra also differ significantly as the cuticle lacks the microtrichia found

14 on the legs of *C. corneri*. The ability of the males to float on the liquid surface by holding

15 their hind legs in a characteristic way, with the flattened and setae-lined areas of the femora,

16 tibia and tarsi resting on the surface of the liquid has parallels in other water-surface-active

17 insect taxa such as pond skaters and relatives (Cheng 1975; Perez Goodwyn et al. 2009).

18

19 Like other pollinator fig wasps, *Ceratosolen* species such as *C. bisulcatus* and *C. corneri*

20 have variable, but usually strongly female-biased offspring sex ratios (Hu et al. 2013; L.J.

21 Rodriguez, unpublished). Mating takes place within the galls of females, and males need

22 to chew entry holes through the walls of each gall to gain entry. The combination of time

1   required to gain access to each female, and the high female to male ratio, appear to have

2   limited the extent to which male pollinators compete directly with each other, and only

3   limited inter-male aggression has been reported (Murray 1989; Nelson and Greeff 2009).

4   Depending on the species, female pollinators may mate only once or mate with several

5   males (Zavodna et al. 2005; Peng et al. 2014), but the time available for mating is typically

6   limited by the short adult life span of the insects. By allowing them to gain earlier access

7   to females, the modified hind legs of *C. corneri* and its relatives provide more time for the

8   males to mate. Males that emerge earlier are also likely to mate with more females, and

9   sexual selection is likely to have favoured the evolution of the modified legs that make this

10   possible.

11

12   Unrelated fig wasps sharing the same figs as *C. corneri* are subject to similar selection

13   pressures, and the hind legs of *C. corneri* exhibit striking functional convergence with the

14   modified peritremata of the male NPFW exhibited by numerous species that also occupy

15   *Sycomorus* figs. *Apocrypta* (Pteromalidae) males have enlarged cup-like peritremata

16   around the metasomal spiracles, and in *Sycophaga* species (Sycophaginae, Agaonidae) the

17   peritremata are drawn out to form elongate, pointed projections (Figure 11). Their

18   peritremata are water-repellent, with similar properties to the legs of *C. corneri,* and

19   similarly allow the males to vacate their galls and seek out females while there is still liquid

20   around their galls (Compton and McLaren 1989). Selection pressures favouring earlier

21   male emergence have therefore generated broadly similar adaptations in these three groups

22   of fig wasps, but the means of achieving this are different. Male pollinators mate using a

23   telescoping metasoma that projects forward beneath the head and is inserted into galls that

1 contain females. Consequently, in order to mate both the head and metasoma need to be

2 inserted below the liquid, if it is present. Mating behaviour among the NPFW is different,

3 and the metasoma does not need to be routinely submerged. Convergence has therefore

4 been constrained, producing structures with rather similar microscopic appearance, but on

5 different parts of the body and protecting different spiracles.

6
7 **Acknowledgements**

15

16 **Disclosure Statement**

17 No potential conflict of interest will arise from the publication of this study.

18
19 **References**
20
21 Alexander AE, Hayter JB. 1971. Determination of surface and interfacial tension. In:
22   Weissberger A, Rossiter BW, editors. Technique of organic chemistry Vol. I:
23   Physical methods of organic chemistry. John Wiley and Sons, Inc. USA; p. 501–
24   512.
25

Balmert A, Bohn HF, Ditsche-Kuru P, Barthlott W. 2011. Dry under water: comparative morphology and functional aspects of air-retaining insect surfaces. J Morphol. 272: 442–451.

Byun D, Hong J, Saputra Ko JH, Lee YJ, Park HC, Byun B-K, Lukes JR. 2009. Wetting characteristics of insect wing surfaces. J Bionic Eng. 6: 63–70.

Chapman RF, Simpson SJ, Douglas AE. 2012. The insects: structure and function. 5th ed. Cambridge University Press, New York.

Chen HH, Yang D-R, Gu D, Compton SG, Peng Y-Q. 2013. Secondary galling: a novel feeding strategy among 'non-pollinating' fig wasps from *Ficus curtipes*. Ecol Entomol. 38: 381–389.

Cheng L. 1975. Insecta Hemiptera: Heteroptera, Gerridae, Genus *Halobates*. Fich. Ident. Zooplancton 147, 4 pp.

Compton SG. 1987. *Aganais speciosa* and *Danaus chrysippus* (Lepidoptera) sabotage the latex defences of their host plants. Ecol Entomol. 12:115–118.

Compton SG, Ball AD, Collinson ME, Hayes P, Rasnitsyn AP, Ross AJ. 2010. Ancient fig wasps indicate at least 34 million years of stasis in their mutualism with fig trees. Biol Letters. 6:838–842.

Compton SG, McLaren FAC. 1989. Respiratory adaptations in some male fig wasps. P K Ned Akad C Biol. 92:57–71.

Cruaud A, Rønsted N, Chantarasuwan B, Chou L-S, Clement WL, Couloux A, Cousins B, Genson G, Harrison RD, Hanson PE, et al. 2012. An extreme case of plant-insect co-diversification: figs and fig-pollinating wasps. Syst Biol. 61:1029–1047.

Dodson CH, Frymire GP. 1961. Natural pollination of orchids. Mo Bot Gard Bull. 49:133–152.

Farache FHA, Rasplus, J-Y, Azar D, Pereira RAS, Compton SG. (in press). First record of a non-pollinating fig wasp (Hymenoptera: Sycophaginae) from Dominican amber, with estimation of the size of its host figs. J Nat Hist.

Galil J, Eisikowitch D. 1968a. Flowering cycles and fruit types of *Ficus sycomorus* in Israel. New Phytol. 67:745–758.

Galil J, Eisikowitch D. 1968b. On the pollination ecology of *Ficus sycomorus i*n East Africa. Ecology 49:259–269.

Galil J, Neeman G. 1977. Pollen transfer and pollination in common fig (*Ficus carica*). New Phytol. 79:163–171.

Guo HY, Li QY, Zhao HP, Zhou K, Feng XQ. 2015. Functional map of biological and
 biomimetic materials with hierarchical surface structures. RSC Advances 5:
 66901–66926.

Holdgate MW. 1955. The wetting of insect cuticles by water. J Exp Biol. 32:591–617.

Hu H-MS, Watson GS, Cribb BW, Watson JA. 2011. Non-wetting wings and legs of the
 cranefly aided by fine structures of the cuticle. J Exp Biol. 214:915–920.

Hu HY, Chen ZZ, Jiang ZF, Huang DW, Niu LM, Fu YG. 2013. Pollinating fig wasp
 Ceratosolen solmsi adjusts the offspring sex ratio to other foundresses. Insect Sci.
 20:228–234.

Janzen DH. 1979. How to be a fig. Annu Rev Ecol Syst. 10:13–51.

Kitching RL. 2000. Food webs and container habitats: the natural history and ecology of
 phytotelmata. Cambridge University Press, New York.

Li Z, Yang P, Peng Y, Yang D. 2009. Ultrastructure of antennal sensilla of female
 Ceratosolen solmsi marchali (Hymenoptera: Chalcidoidea: Agaonidae:
 Agaoninae). Can Entomol. 141:463–477.

Matheson T. 2013. Mechanoreception. In: Chapman RF, author. The Insects: Structure
 and Function. 5th ed. Cambridge University Press; pp. 738–770.

Moe AM, Weiblen GD. 2010. Molecular divergence and host conservatism in
 Ceratosolen (Agaonidae) pollinators of geographically widespread *Ficus* species
 (Moraceae). Ann Entomol Soc Am. 103:1025–1037.

Monteiro RF, Macedo MV. 2014. First report on the diversity of insects trapped by a
 sticky exudate of the inflorescences of *Vriesea bituminosa* Wawra (Bromeliaceae:
 Tillandsioideae). Arthropod Plant Inte. 8:519–523.

Murray MG 1989. Environmental constraints on fighting in flightless male fig wasps.
 Anim Behav. 38:186–193.

Nakanishi A, Nishino H, Watanabe H, Yokohari F, Nishikawa M. 2009. Sex-specific
 antennal sensory system in the ant *Camponotus japonicus*: structure and
 distribution of sensilla on the flagellum. Cell Tissue Res. 338:79–97.

Nelson RM, Greeff JM. 2009. Male pollinator fig wasps (Chalcidoidea: Agaonidae) do
 not need an arena to fight. Afr Entomol. 17:228–231.

Pedersen O. 2012. Physical gills prevent drowning of many wetland insects, spiders and
 plants. J Exp Biol. 215:705–709.

Peng Y-Q, Zhang Y, Compton SG, Yang D-R. 2014. Fig wasps from the centre of figs have more chances to mate, more offspring and more female-biased offspring sex ratios. Anim Behav. 98: 19–25.

Perez Goodwyn P, Maezono Y, Takamatsu H, Fujisaki K. 2009. Semiaquatic Heteroptera locomotion: coral treaders (*Hermatobates weddi*, Hermatobatidae), sea skaters (*Halovelia septentrionalis*, Veliidae), and water striders (*Metrocoris histrio*, Gerridae). Usual and unusual gaits. Hydrobiologia. 630:219–229.

Ravaiano SV, Ferreira RdP, Campos LAdO, Martins GF. 2014. The antennal sensilla of *Melipona quadrifasciata* (Hymenoptera: Apidae: Meliponini): a study of different sexes and castes. Naturwissenschaften. 101:603–611.

Ramirez W. 1996. Breathing adaptations of males in fig gall flowers (Hymenoptera: Agaonidae). Rev Biol Trop. 44/45:277–282.

Richards AG, Richards PA. 1979. The cuticular protuberances of insects. Int J Insect Morphol. 8:143–157.

Stankiewicz BA, Poinar HN, Briggs DEG, Evershed RP, Poinar GO. 1998. Chemical preservation of plants and insects in natural resins. P Roy Soc Lond B Bio. 265:641–647.

Suleman N, Raja S, Compton SG. 2012. Only pollinator fig wasps have males that collaborate to release their females from figs of an Asian fig tree. Biol Letters. 8: 344–346.

Tuteja A, Choi W, Ma M, Mabry JM, Mazzella SA, Rutledge GC, McKinley GH, Cohen RE. 2007. Designing superoleophobic surfaces. Science: 318(5856):1618–22.

Vazquez G, Alvarez E, Navaza JM. 1995. Surface-tension of alcohol plus water from 20-degrees-C to 50-degrees-C. J Chem Eng Data. 40:611–614.

Weiblen GD. 2002. How to be a fig wasp. Annu Rev Entomol. 47:299–330.

Wiebes JT. 1994. Agaonidae (Hymenoptera Chalcidoidea) and *Ficus* (Moraceae): fig wasps and their figs, XIII (*Ceratosolen* & additions). Proc Kon Ned Akad Wet Ser C. 97:123–136.

Zavodna M, Compton SG, Raja S, Gilmartin PM, van Damme J. 2005. Do fig wasps produce mixed paternity clutches? J Insect Behav. 18:351–362.

Zhang X-M, Wang S, Li S, Luo C, Li YX, Zhang F. 2015. Comparison of the antennal sensilla ultrastructure of two cryptic species in *Bemisia tabaci*. PLoS ONE. 10: e0121820.

Figure 1.

Figure 2.

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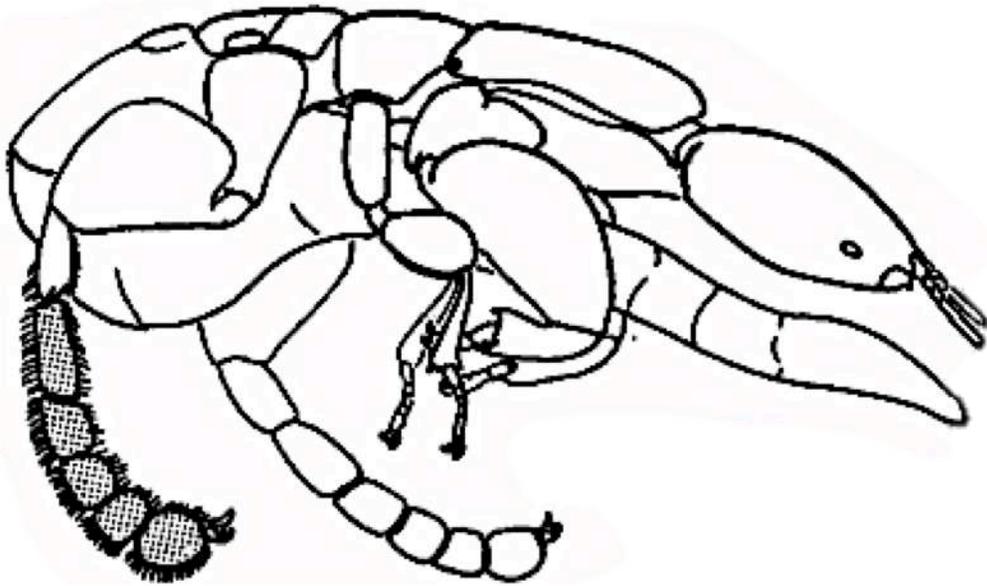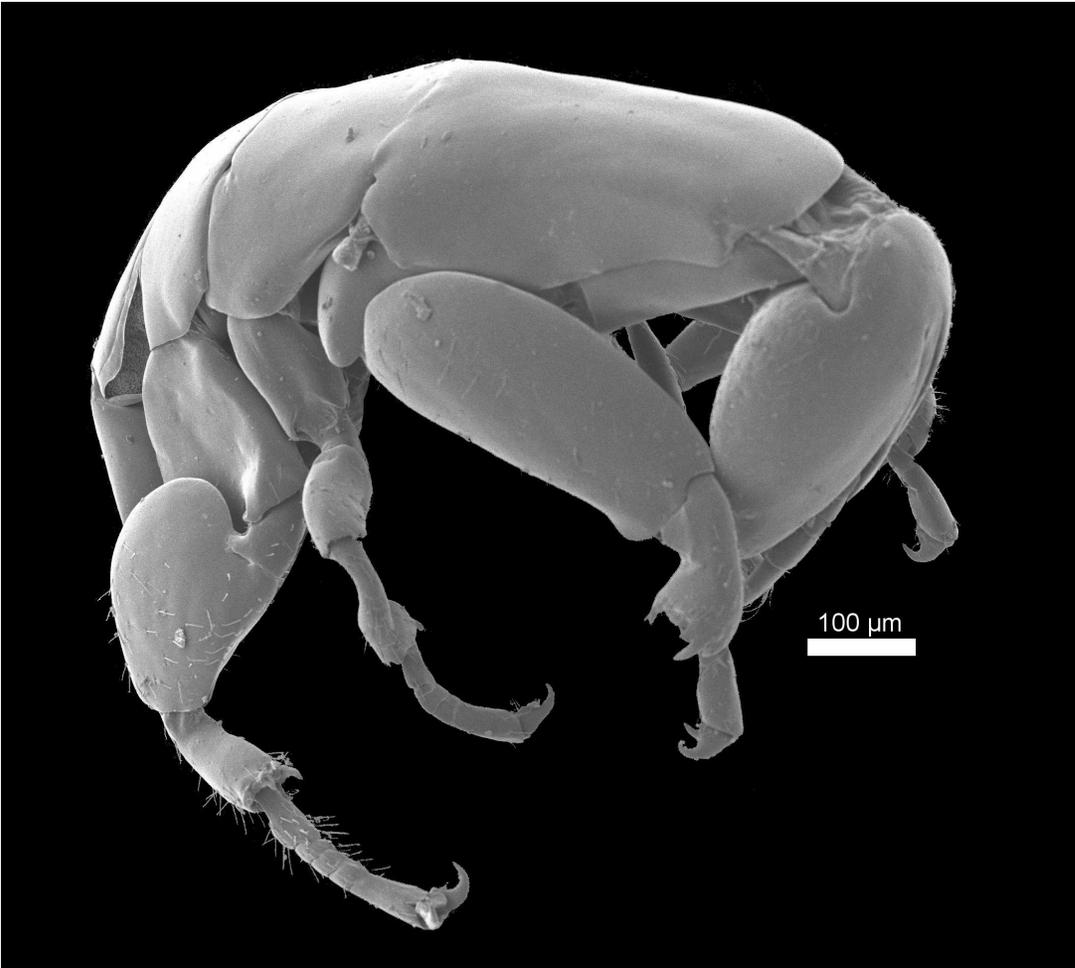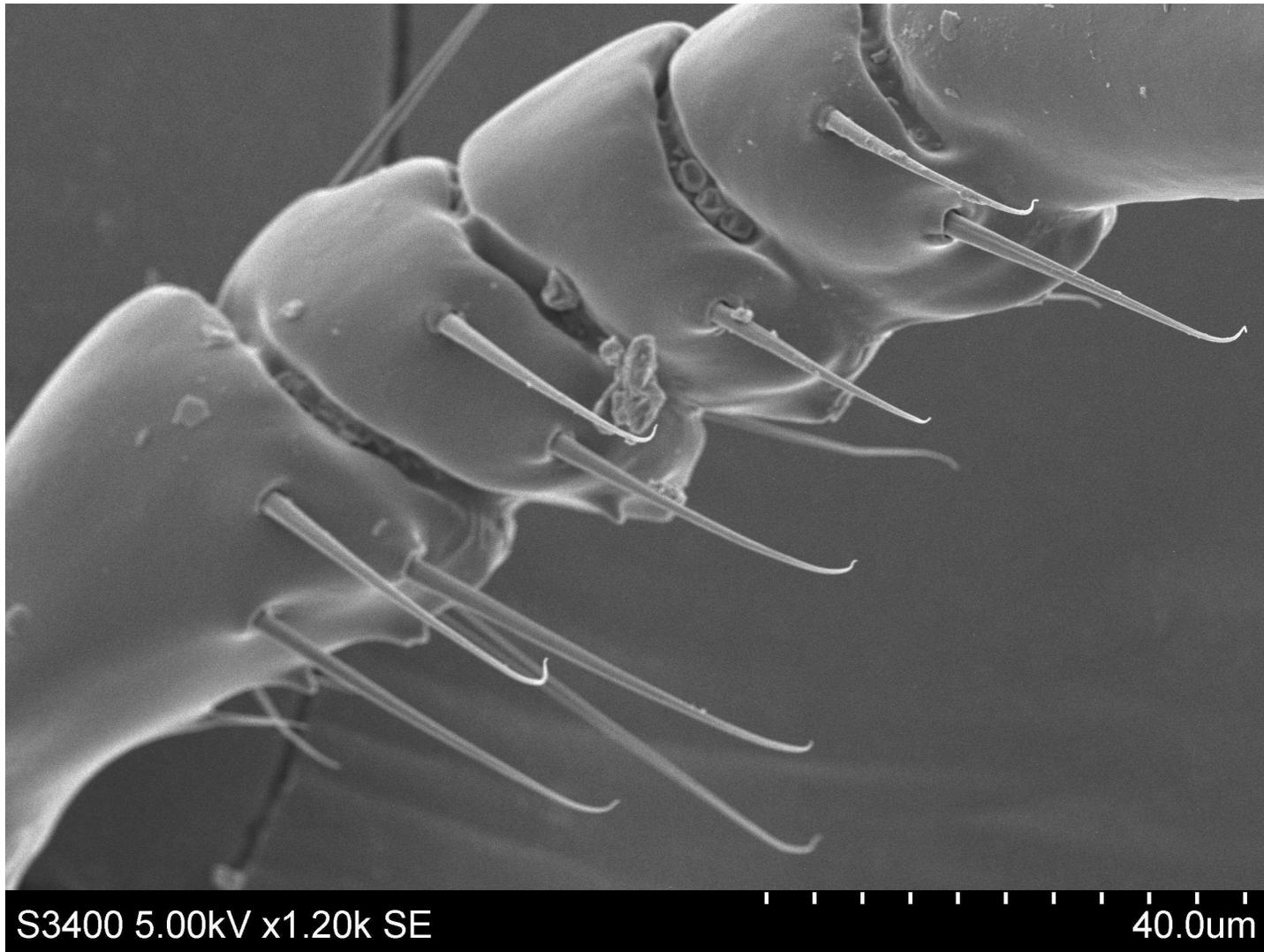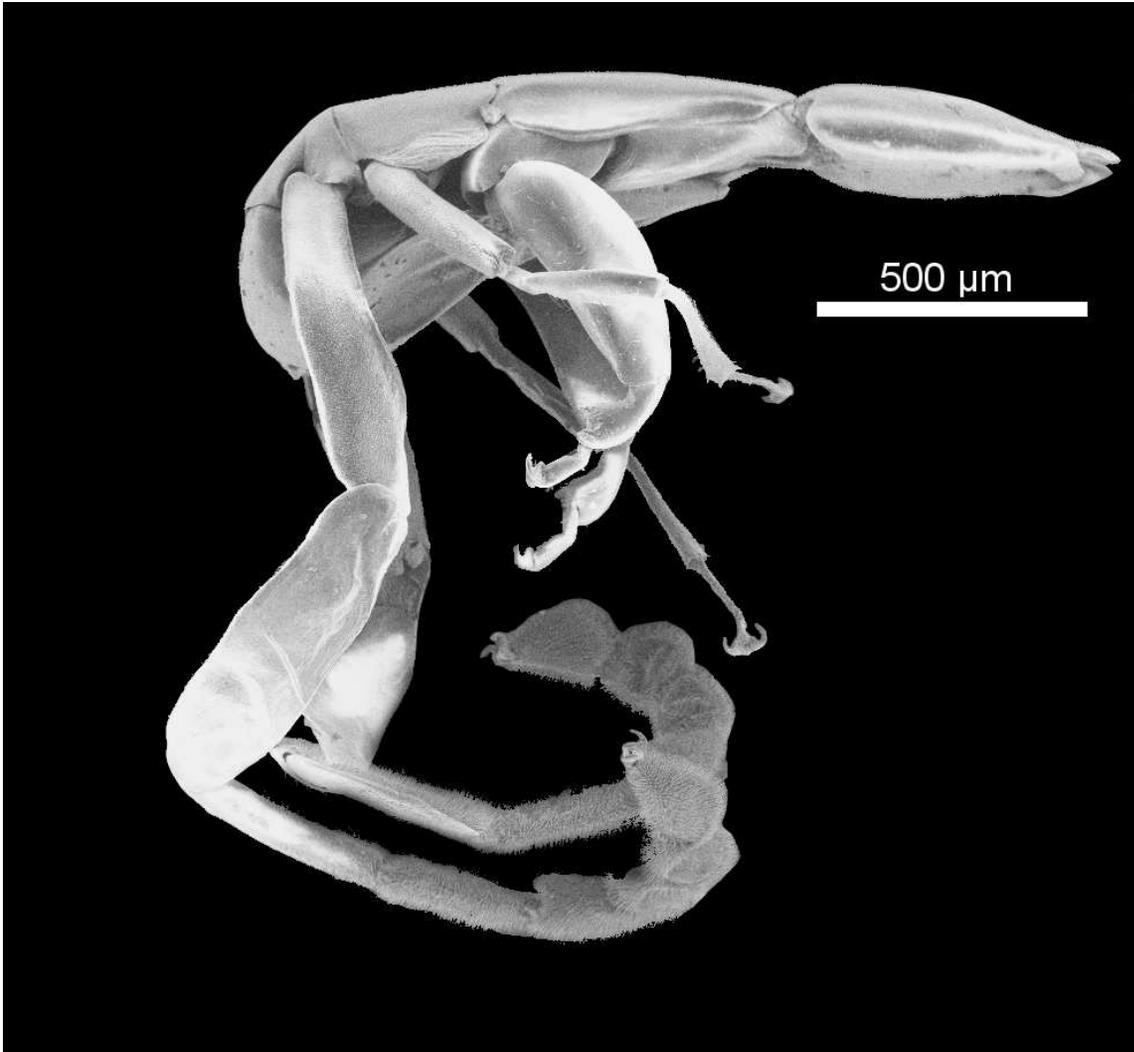
Figure 3.

Figure 4.

Figure 5.

Figure 6.

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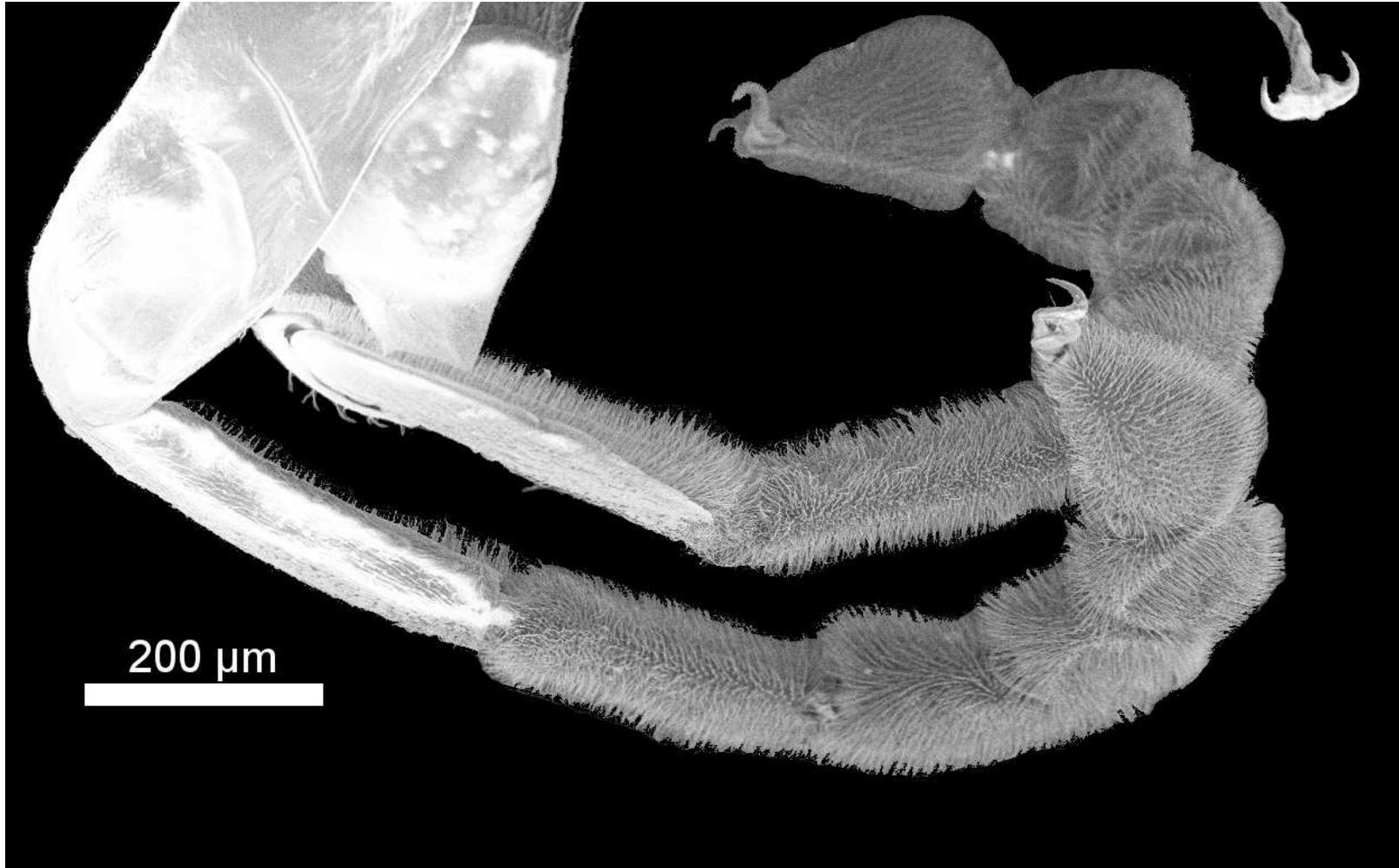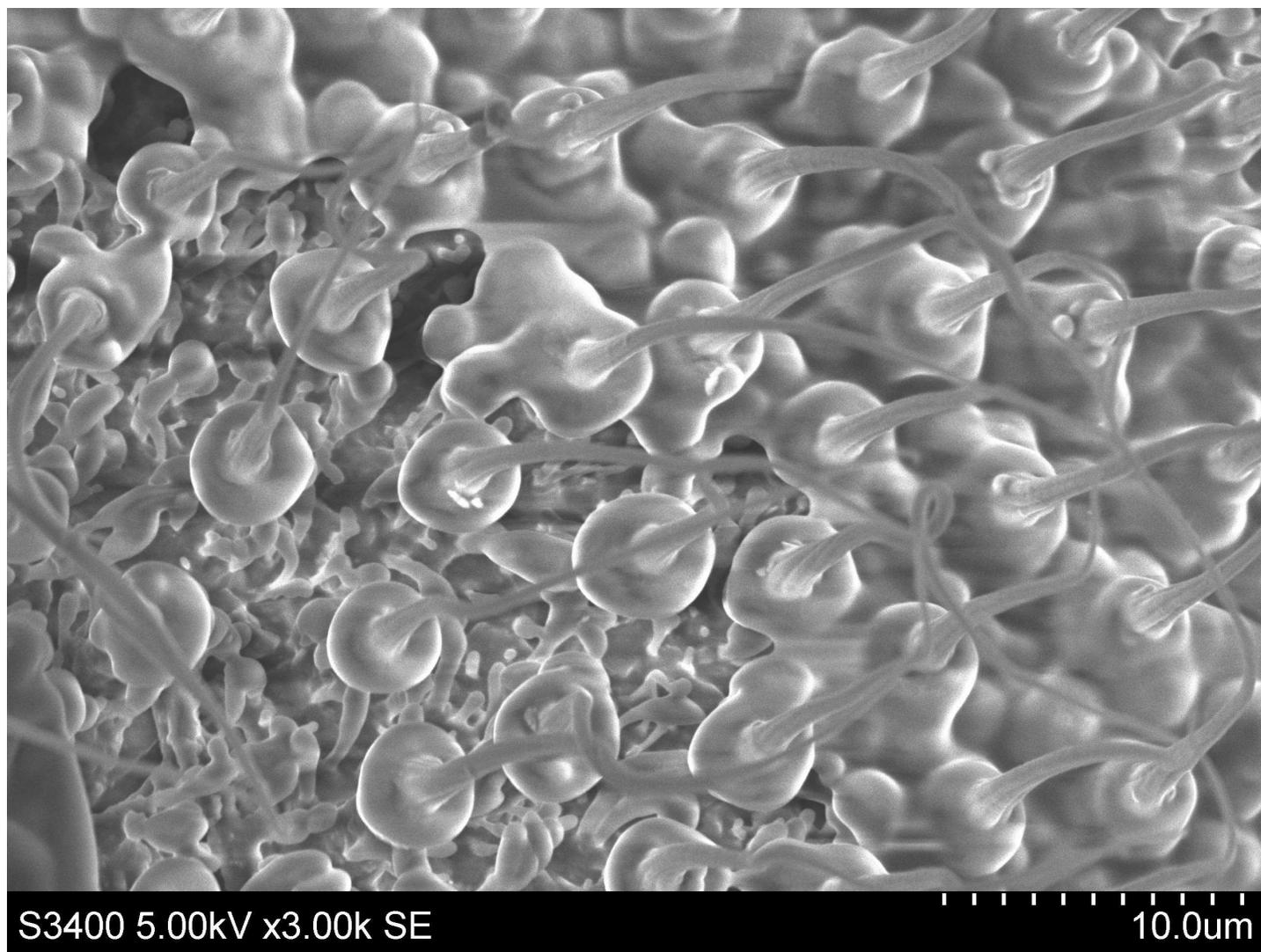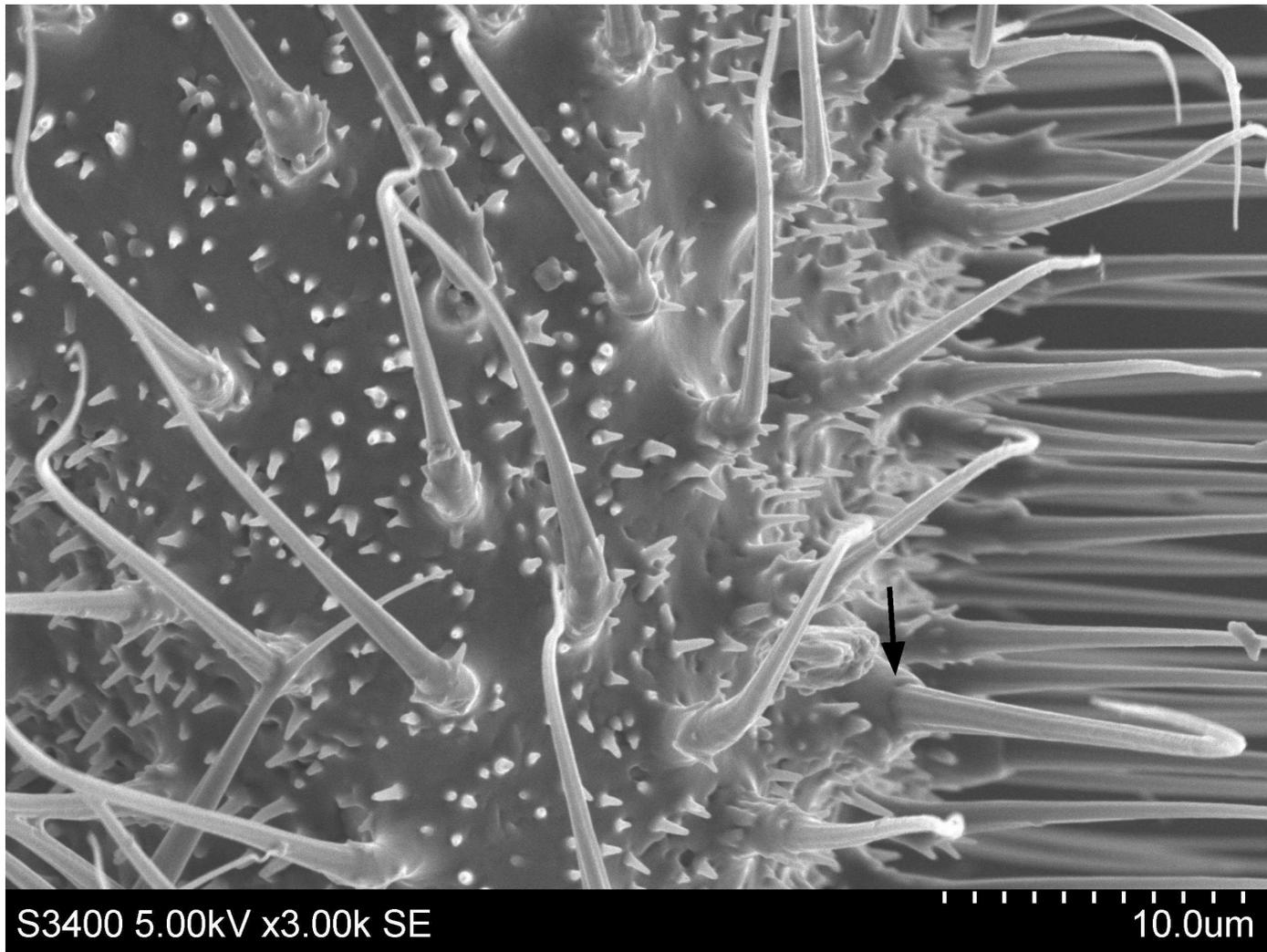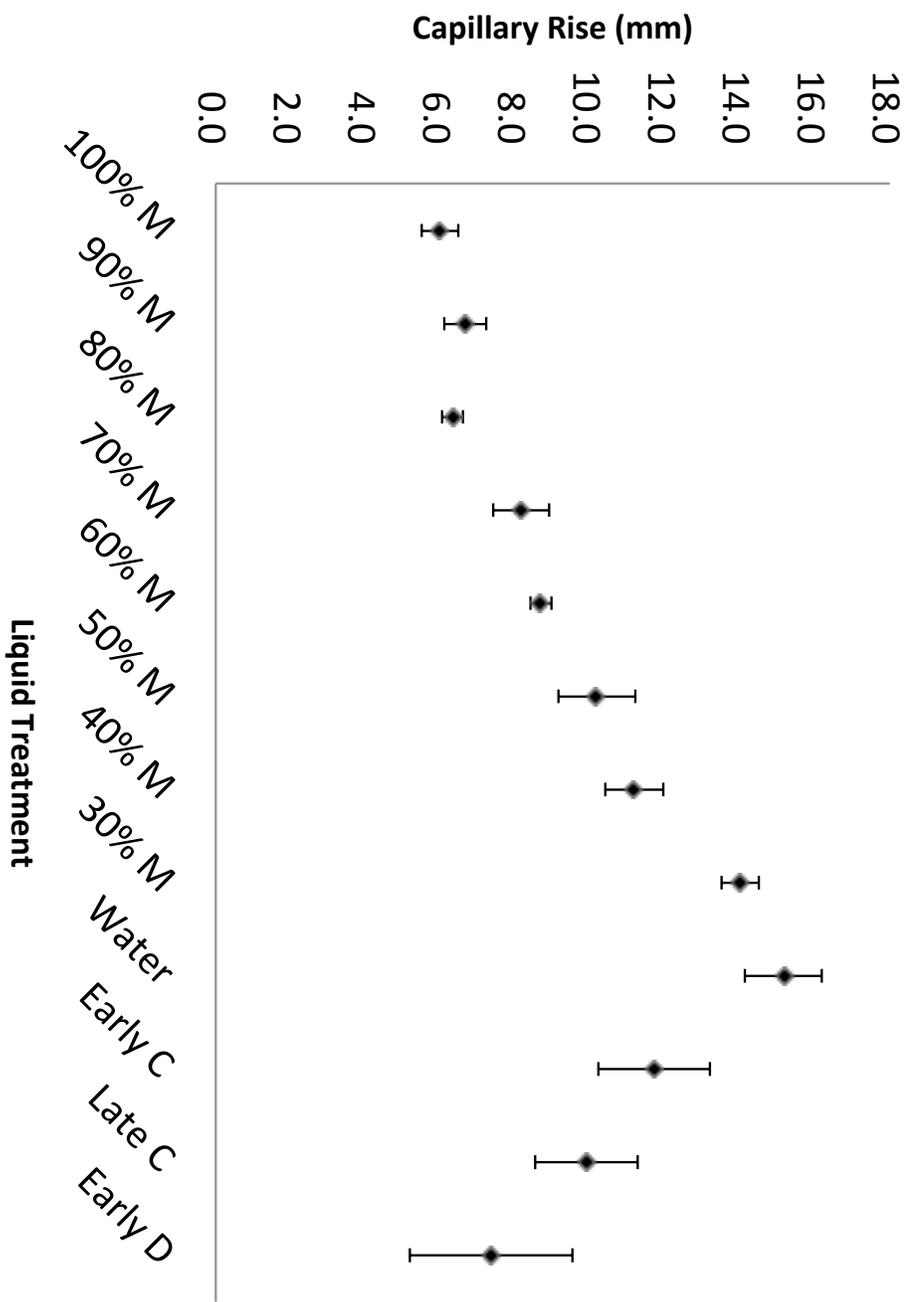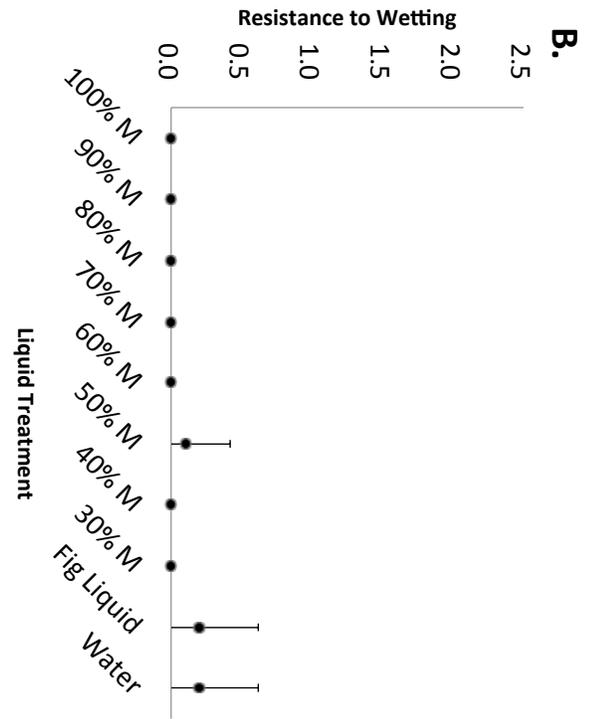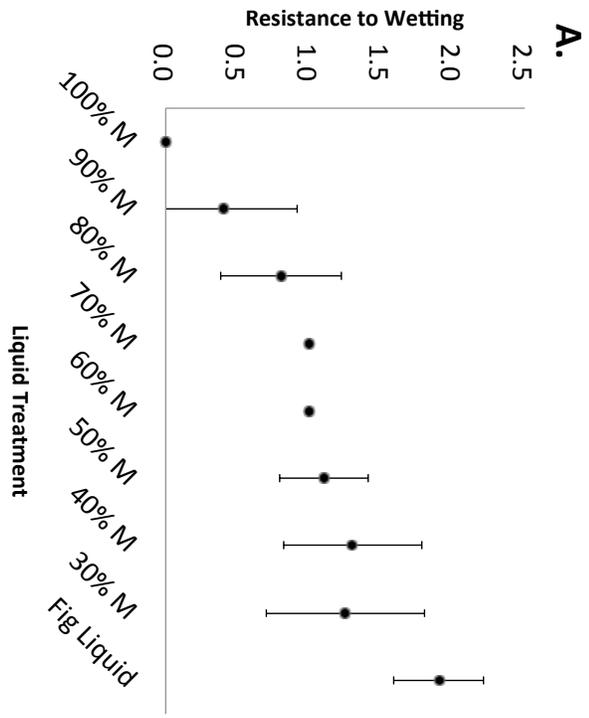
Figure 7.

Figure 8.

Figure 9.

Figure 10.

Table 1. Variation in setae length, density and distances apart and microtrichia length between the different hind leg segments of *Ceratosolen bisulcatus* and *Ceratosolen corneri*. Values presented are means ± SD. Dashes indicate absence from specified segments.

Species	Hind leg segments	Non-socketed setae Lengths (µm)	Non-socketed setae density (setae/mm²)	Distance between non-socketed setae (µm)	Socketed setae lengths (µm)	Socketed setae density (setae/mm²)	Microtrichia lengths (µm)
C. bisulcatus	Tarsus 5	-	-	-	28.17 ± 3.91	2055.4	-
	Tarsus 4	-	-	-	29.72	1963.6	-
	Tarsus 3	-	-	-	28.79	1930.3	-
	Tarsus 2	-	-	-	36.77 ± 5.39	3224.2	-
	Tarsus 1	-	-	-	37.07 ± 6.00	1262.7	-
	Tibia	-	-	-	21.27 ± 3.22	2836.4	-
	Femur	-	-	-	19.07 ± 2.88	1796.2	-
	Coxa	-	-	-	-	-	-
C. corneri	Tarsus 5	23.60 ± 9.04	21567.2 ± 8968.9	5.79 ± 1.64	27.78 ± 6.90	459.3	0.71 ± 0.33
	Tarsus 4	19.79 ± 8.00	17573.0 ± 4196.3	5.41 ± 1.94	17.34	269.6	0.73 ± 0.33
	Tarsus 3	19.41 ± 4.15	13763.4 ± 2344.8	5.86 ± 1.57	27.16 ± 8.73	1052.6	0.95 ± 0.36
	Tarsus 2	18.27 ± 3.99	8563.6 ± 6264.5	5.67 ± 1.45	29.15	1856.2	0.77 ± 0.36
	Tarsus 1	16.58 ± 2.23	28740.1 ± 571.4	3.88 ± 0.98	-	-	0.62 ± 0.22
	Tibia	14.17 ± 2.27	37449.8 ± 6125.6	3.24 ± 0.80	-	-	0.73 ± 0.27
	Femur	14.79 ± 6.97	31102.1 ± 163.5	3.89 ± 1.21	-	-	0.94 ± 0.47
	Coxa	18.30 ± 5.66	31767.3 ± 3652.7	3.71 ± 0.64	-	-	1.04 ± 0.39

Table 2. Adult male pollinator fig wasp activity in relation to the amounts of liquid present in the lumen of three *Ficus* species. *Ceratosolen corneri* is the pollinator of both *Ficus linearifolia* and *Ficus botryocarpa*. *Ficus septica* is pollinated by *Ceratosolen bisulcatus*. *F*- and *P*-values are from Generalized Linear Models with Quasi-Poisson Error distributions.

Liquid present	*Ficus linearifolia*		*Ficus botryocarpa*		*Ficus septica*	
	N figs	Active males (Mean ± SD)	N figs	Active males (Mean ± SD)	N figs	Active males (Mean ± SD)
> 50% of cavity	7	1.00 ± 1.53	7	35.71 ± 24.40	5	0
< 50% of cavity	4	1.00 ± 0.82	5	40.00 ± 22.36	6	0
Flowers covered	6	8.50 ± 9.44	3	33.33 ± 28.87	5	0
Ovules and seeds covered	6	8.83 ± 6.85	6	25.00 ± 27.39	2	1.00 ± 1.41
No liquid	8	8.75 ± 5.97	10	50.00 ± 0	12	16.00 ± 17. 9
F		32.47		1707		12.5
P		<0.0001		<0.0001		<0.0001

Figure Legends

Figure 1. Drawing of a *Ceratosolen dentifer* Wiebes adult male showing the elongate hairy hind legs and the forward-pointing telescopic gaster extending forward beneath the head (from Wiebes 1994, Figure 2, with permission).

Figure 2. Scanning electron micrograph of a *Ceratosolen bisulcatus* adult male, showing the short and sparsely-hairy hind legs.

Figure 3. Scanning electron micrograph of the hind leg tarsal segments of *Ceratosolen bisulcatus* showing the socketed trichoid setae with grooved-surfaces and bent tips.

Figure 4. Scanning electron micrograph of an adult male *Ceratosolen corneri* (from *Ficus botryocarpa*) showing the elongate hind legs and the metasoma curved forwards beneath the thorax. It is extended further, in front of the head, during mating.

Figure 5. The hind legs of an adult male *Ceratosolen corneri* (from *Ficus botryocarpa*) showing the recurved elongate and densely hairy tarsi and the hairy inner face of the tibiae.

Figure 6. Scanning electron micrograph of the proximal part of the inner face of the hind coxa of *Ceratosolen corneri* (from *Ficus botryocarpa*) showing the non-socketed setae with raised bases and the highly modified cuticular surface.

Figure 7. Scanning electron micrograph of the fourth tarsal segment on the hind legs of *Ceratosolen corneri* (from *Ficus botryocarpa*) showing the socketed setae (black arrow), the more numerous non-socketed setae and short microtrichia.

Figure 8. The surface tension (mean ± SD) of the liquid inside figs of *Ficus botryocarpa* at developmental stages C and D, as measured by capillary rise, compared with distilled water and methanol dilutions.

Figure 9. Variation in resistance to wetting (means ± SD) of male fig wasp hind legs in relation to immersion in liquids with differing surface tensions. The solutions are ordered in sequence of increasing surface tension. Resistance to wetting was scored using an index of 0 (least resistant), 1 or 2 (most resistant), depending on the formation of air bubbles between the hind legs. (A) *Ceratosolen corneri* from *Ficus botryocarpa*. (B) *Ceratosolen bisulcatus* from *Ficus septica*.

Figure 10. Resting position of the hind legs of a male *Ceratosolen corneri* when floating in a head-down position on the surface of distilled water. The water-repellent legs ensured that the propodeal spriracles (anterior to the base of the gaster) remained clear of the water surface.

Figure 11. Scanning electron micrograph of *Sycophaga fusca* showing the (A) metasoma and (B) modified peritremata towards the end of the metasoma typical of the males of this genus.