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| Citation         | Journal of the Royal Society interface, 20(205)<br><a href="https://doi.org/10.1098/rsif.2023.0324">https://doi.org/10.1098/rsif.2023.0324</a>                     |
| Issue Date       | 2023-08-16   |
| Doc URL          | <a href="http://hdl.handle.net/2115/90553">http://hdl.handle.net/2115/90553</a>  |
| Type             | article (author version)   |
| File Information | rsif_2023.0324.pdf   |



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# 1 The tight attachment achieved by the male discoidal setae is possibly a counter-adaptation 2 to the grease layer on female integument surfaces in green dock beetles

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8

## 9 Abstract

10 Green dock beetles *Gastrophysa viridula* exhibit sexual dimorphism in tarsal attachment setae: females  
11 have only pointed, lanceolate, and spatula-like setae, while males additionally possess discoidal ones. The  
12 sexual dimorphism is likely attributed to the necessity of male discoidal setae to adhere to the smooth back  
13 of the female during copulation. We aimed to understand its possible mechanism of attachment with *G.*  
14 *viridula*. Pull-off forces of both females and males were measured on (1) alive females, (2) dead and dried  
15 females, and (3) resin replicas of fresh females. The attachment ability tended to increase on dead and  
16 replicated female surfaces in both sexes, which indicates that the epicuticular grease layer on the  
17 integument of alive intact beetles decreases the attachment. This tendency was prominent in females. The  
18 present study clearly showed that in *G. viridula* discoidal setae enable the males to adhere stronger to  
19 female surfaces. The divergent performance found between the sexes differing in their setal composition is  
20 likely caused by the stiffness difference between the setae types and by the specific shape of the setal tips.  
21 A peculiar reproductive biology in *G. viridula* is likely attributed to this remarkable divergence of labour  
22 in their attachment pads between the sexes.

23

24 Keywords: adhesion, copulation, insect, sexual dimorphism

25

## 26 1. Introduction

27 Fibrillar attachment structures have evolved independently in many insects and arachnids [1-5], which  
28 enables them to adhere to a wide spectrum of surfaces in nature and expand their habitats. Considering  
29 that attachment organs for locomotion are one of the most essential interfaces between organisms and their  
30 environment, the evolution of these organs was crucial for many arthropods that prowl or crawl on various  
31 substrata. The tip shape of these fibrillar structures is diverse among species and even within a single  
32 attachment pad [4, 6-8], resulting in a wide variety of terrestrial and aquatic surfaces where animals can  
33 walk on and adhere to.

34 Not exclusively, but in many beetles, sexual dimorphism of attachment pads is well known: females have  
35 pointed, lanceolate, and spatula-shaped setae, while males possess discoidal setae as well [4, 6]. The  
36 evolutionary background of the origins of this sexual dimorphism has been considered that males can better  
37 adhere to female smooth and greasy elytra surfaces during copulation due to the presence of the discoidal  
38 setae, despite the potential hindrance of this setal type on rough substrates [9-13]. Voigt et al. [14] presented  
39 the first empirical evidence supporting this hypothesis for the leaf beetle *Chrysolina americana* in vivo.  
40 Another indirect evidence comes from the experimental study on the ladybird beetle *Cryptolaemus*  
41 *montrouzieri* [15]. In this species, hairy attachment structures of both females and males do not exhibit  
42 sexual dimorphism and do not significantly differ in their attachment forces between the sexes. In the case

43 of the latter species, the female elytral surface is entirely covered with long setae that presumably prevent  
44 males from adhering to this surface, and the males are rather prone to interlock to the elytral edges using  
45 their claws [15].

46 The tarsal attachment in insects belongs to the so-called ‘wet adhesion’ type, since tarsal structures  
47 produce an emulsive secretion [4, 16]. The entire body surface of an insect is covered with a lipid-based  
48 liquid [12], which content is partially congruent with the tarsal secretion [8, 17-20]. Without this secretion,  
49 the attachment force decreases [21-23]. Thus, the contribution of the secretion to the tarsal adhesion is  
50 crucial without any doubt. On the other hand, the increase of the fluid layer thickness could switch the  
51 function of the fluid from an increase of adhesive and friction forces to a lubrication. Due to this effect, the  
52 grease layer on the female body surface could reduce tarsal adhesion [12, 24, 25]. Moreover, stiffness,  
53 chemistry, texture, and humidity of substrates are also known to influence attachment forces [7, 9, 26-37].  
54 Until now, these effects have been well studied on artificial flat smooth and rough surfaces, but rarely in the  
55 context of copulation that never occurs on flat surfaces.

56 In the present study, we chose the green dock beetle *Gastrophysa viridula* whose tarsal adhesion has  
57 been intensively studied [9, 10, 18, 22, 25, 26, 33, 35, 36, 38-45], to unveil the impact of the surface  
58 material and grease on its adhesion during copulation. Bullock and Federle [9] have previously measured  
59 friction and adhesive forces of beetles of both sexes on removed female elytra. However, in the case of  
60 this species, the elytra are not a relevant body region for copulation. Although they show the typical  
61 mating posture of leaf beetles, i.e. the male-above position [46], the *G. viridula* female has a huge swollen  
62 abdomen exposed from the elytra during a reproductive season, and males adhere to the exposed abdomen  
63 to mate [47], unlike *C. americana*, the only other species, whose attachment forces were measured on  
64 alive females [14]. Considering that substrate material and especially its stiffness plays an important role  
65 in attachment [27, 28, 33], the highly sclerotised elytra compared to the membranous abdominal sternite  
66 might affect the attachment ability. Our study aims to address the following questions: (1) does the sexual  
67 dimorphism in tarsal attachment structures affect their adhesion performance in the context of mating  
68 behaviour in *G. viridula*? (2) does a removal of the grease layer affect adhesion? (3) does substrate  
69 material affect adhesion? For this purpose, we performed attachment force measurement on alive and dead  
70 (dry) females as well as on females moulded of resin.

71

## 72 **2. Materials and methods**

### 73 (a) Beetles

74 All adults of *G. viridula* (De Geer, 1775) used for the study were collected on the campus of Kiel  
75 University and used for observations and experiments. Some of them were kept in a small plastic box for a  
76 few days with their host plants, *Rumex* spp. at room temperature without any temperature, light, and  
77 humidity control before being used for force measurements described below. Therefore, no individual  
78 records on age and mating experience are available. However, only intact beetles during inspections were  
79 included.

80

### 81 (b) Cryo-scanning electron microscopy (Cryo-SEM)

82 To visualise interactions between male tenant setae and the female abdomen, Cryo-SEM was applied to  
83 two coupling beetle pairs. Two coupling pairs were instantly fixed with liquid nitrogen at ca. -200 °C and  
84 immediately transferred to a chamber of the cryo-preparation system Gatan ALTO 2500 (Gatan Inc.,  
85 Abingdon, UK) (-140 °C) of SEM Hitachi S-4800 (Hitachi High-Technologies Corp., Tokyo, Japan).

86 Water crystals covering the sample surfaces were sublimated by raising the temperature (-120 °C) in the  
87 preparation chamber. Subsequently, the chamber temperature was set to -140 °C. Then, the samples were  
88 sputter-coated in the frozen condition with gold-palladium (thickness 10 nm), transferred to the SEM main  
89 chamber, and examined at 3 kV acceleration.

90

#### 91 (c) Force measurement: substrate preparation

92 For measuring attachment forces, 36 sexually matured females were used as either of the following three  
93 substrates: (1) alive females, (2) dead females, and (3) resin replicas of females (Figure 1a-c). In the first  
94 group, 12 alive females were anaesthetised with CO<sub>2</sub> and glued on SEM sample stubs with  
95 polyvinylsiloxane polymer (PVS) (Coltène® PRESIDENT light body, Coltène/Whaledent AG, Altstätten,  
96 Switzerland) immediately before starting every force measurement (Figure 1a). The SEM specimen stubs  
97 were placed on a commercial dental wax mass on a laboratory scissor jack so that the angle of focus  
98 females could be adjusted properly. To remove the epicuticular grease layer, other 12 females were  
99 anaesthetised and submerged in 2.5 % glutaraldehyde in phosphate-buffered saline (PBS; pH = 7.4; Carl  
100 Roth GmbH & Co. KG, Karlsruhe, Germany) for ca. 16 hours. Then, the female samples were rinsed with  
101 PBS and dehydrated in an ascending series of ethanol, till absolute ethanol. Subsequently, they were dried  
102 using a critical point dryer (CPDA/Quorum Technologies Ltd., Kent, UK) and glued on SEM sample  
103 stubs as described above (Figure 1b). For the preparation of resin females, the other 12 females prepared  
104 in a conventional freezer were moulded using PVS. After the females were removed, Epon 812  
105 (Glycidether 100; Carl Roth GmbH & Co. KG) was poured into the moulds (Figure 1c). The Epon was  
106 polymerised at 60 °C for 48 h, and the resin females thus obtained were glued as the others (Figure 1c).

107

#### 108 (d) Force measurement: experiment

109 Attachment force measurement was conducted with 12 females and 12 males using a pull-off force  
110 experimental set-up consisting of a 100 g force sensor FORT-100 (World Precision Instruments, Sarasota,  
111 FL, USA) connected to a force transducer MP 100 (Biopac Systems, Goleta, CA, USA). One day before  
112 experimenting, beetles were anaesthetised with CO<sub>2</sub>, and their claws were cut off using a micro-scissor.  
113 Since cutting the claws completely at the very basis could damage the tendon, which is connected to the  
114 unguitractor plate [47], only tip regions were removed (Figure S1). However, such claws could still hook  
115 the elytron edge of the females used as substrates as described below (Figure 1e (left)). The 24  
116 experimental beetles were treated as 12 sets of one female and one male for further experiments. The  
117 beetles' elytra were glued to an insect pin with bee wax, and the pins were firmly fixed to the sensor tip.  
118 The female and male feet were brought into contact with a substrate, kept in contact for a few seconds  
119 until tested beetles calmed down on each surface, and then pulled off at 200 μm/sec. using a DC 3001R  
120 manipulator (World Precision Instruments, USA), to which the force sensor was bound.

121 Prior to each set of measurements, experimental females and males were weighted using a Mettler  
122 Toledo AG 204 Delta Range micro-balance (Greifensee, Switzerland). A set of one alive female, one dead  
123 female, and one resin replica of a female was assigned to each set of one experimental female and one  
124 experimental male, and pull-off forces were measured twice, on both the abdomen and elytra (Figure 1e).  
125 All measurements were conducted with beetles in the right-side-up position. Experiments were carried out  
126 at  $24.8 \pm 2.4$  °C and  $49.0 \pm 7.1$  % relative humidity. To avoid a possible effect of experimental orders on  
127 results of force measurements, the order of the substrates was randomised among the 12 sets (Table S1).  
128 Force-time curves (Figure 1f) were recorded for all experiments and processed using the AcqKnowledge

129 3.7.0 software (Biopac Systems Ltd, Goleta, CA, USA). For some sets of experiments, high-speed camera  
130 recording was also conducted.

131 Four dead females and four resin replicas used for the force measurements were sputter-coated with  
132 gold-palladium (thickness 12 nm) using a magnetron sputter coater E-1030 (Hitachi High-Technologies  
133 Corp.). Subsequently, their surfaces were examined with a SEM JSM-6301F (JEOL, Tokyo, Japan) at 5  
134 kV acceleration.

135

#### 136 (e) Grease visualisation

137 After the force experiments, both alive females and dead females utilised as substrates were examined  
138 using the Cryo-SEM. To ease the visualisation of the grease thickness on these female surfaces, the  
139 females were powdered with a tiny amount of small glass beads, dry borosilicate glass microspheres (ca.  
140 400 nm in diameter) (Duke Scientific Corporation, Palo Alto, CA, USA) using fine tweezers.  
141 Subsequently, the samples were instantly fixed in liquid nitrogen and immediately transferred to a cryo-  
142 preparation chamber. To visualise liquids covering the surfaces, we also fractured the samples using a cold  
143 (-140 °C) scalpel in the cryo-preparation chamber of the Cryo-SEM and observed the fractures after  
144 sublimation and sputter-coating as described above (section (b)). For taking micrographs of epidermal  
145 grease layers to estimate their thicknesses, the sublimation process was omitted. Since the epicuticular  
146 grease layers were not aligned well perpendicular to a SEM-detector, those measurements are rough  
147 estimations.

148

#### 149 (f) Statistics

150 Data analyses and plotting were conducted with R (v.4.2.0) in RStudio [49]. The effects of explanatory  
151 factors (fixed effects) on the pull-off forces were analysed by fitting linear mixed effects models using the  
152 ‘lmer()’ function in the ‘lme4’ package [50]. A normal error structure and an identity link function were  
153 used. Each beetle was measured twice on each surface, therefore individuals were included with a random  
154 factor (only an intercept) in the models. As explanatory factors, (1) sex (female, male), (2) condition  
155 (alive, dead, resin replica), and (3) position (abdomen, elytra) were incorporated. To choose the best  
156 combination of the fixed effects in terms of goodness of prediction, the Akaike information criterion  
157 (AIC) was calculated for the following models: (a) models incorporating three fixed effects but no  
158 interactions of them, (b) models incorporating three fixed effects and one of the possible combinations of  
159 interactions between two fixed effects, (c) models incorporating three fixed effects and one of possible  
160 three combinations of interactions between two fixed effects, (d) models incorporating three fixed effects  
161 and all possible three combinations of interactions between two fixed effects, and (e) model (d) plus the  
162 interaction of three fixed effects (full model). Female and male data were compared within each  
163 combination of conditions and positions using Welch’s t-test, after the normal distribution was tested with  
164 the Shapiro-Wilk test and homoscedasticity was tested with the F-test. All data were plotted using the  
165 ‘ggplot2’ package [51].

166

### 167 3. Results

#### 168 (a) Cryo-fixated couples

169 Although both beetle couples were successfully fixed, while a male was mounting a female, only one  
170 male retained the penetrated aedeagus in the partner’s vagina (Figure 2a). In both cases, a typical mating

171 posture of the study species was observed, in which males hold a female swollen abdomen with their six  
172 tarsi (Figure 2a). In one couple, the male seemingly slipped on the female abdomen, where some traces of  
173 male adhesive setae were detected (Figure 2b,c). The other couple ended the copulation during the fixation  
174 procedure, but the male remained attached to the female using all his tarsi: at least the right foretarsus was  
175 situated on the edge of the female elytron, whereas the other ones attached to the female abdominal  
176 sternite (Figure S2).

177

## 178 (b) Pull-off forces

179 All females and males used for the force measurements on experimental substrates ceased to move their  
180 legs vigorously as soon as they were placed. After a few seconds of a reassuring time, they were lifted  
181 using a micromanipulator. While being lifted, the beetles repeatedly moved all legs toward the body  
182 (Videos S1-12), which brought several peaks in force curves (Figure 1f), and the largest peak was taken as  
183 a pull-off force. In some cases, the claws hooked the elytron edge of alive or dead females; such  
184 measurements were terminated immediately after hooking the elytron edge was detected and/or a large  
185 sudden peak in force curves was observed. When the measurement was interrupted, the third measurement  
186 was performed and treated as the second one (mentioned in Table S2 as comments). All data are  
187 accommodated in Tables S2-4.

188 The first and the second measurements were highly correlated (Pearson correlation coefficients: 0.81 for  
189 females and 0.89 for males) and the OLS regression coefficients were 0.96 (95% confidence interval:  
190 0.79, 1.14) and 0.93 (0.81, 1.04) for females and males, respectively (Figure S3). For the female data, two  
191 data points showed deviation towards the upper side of the regression line (Figure S3), meaning that these  
192 second measurements dropped largely in comparison to the corresponding first ones. The regression  
193 coefficient of these data without the outliers was 0.92 (0.79, 1.05), and the correlation coefficient was 0.87  
194 (Figure S4). Therefore, we considered that the repeatability of the measurements is fulfilled. The  
195 measurements successively obtained on the same substrates were averaged and used for further analyses.  
196 We conducted statistical analyses for all measurements and for the data, where the two female outliers  
197 were excluded (the outliers are highlighted in Table S2 and Figure S3). Only the results based on all data  
198 are shown below, since the obtained results were congruent (compare Tables S5, S7, S9, 2, Figure 3 with  
199 Tables S6, S8, S10, S12, Figure S6 respectively).

200 Pull-off forces ranged from 1.32 to 28.78 mN ( $9.21 \pm 5.40$ , mean  $\pm$  sd) for females and 2.31 to 26.19  
201 mN ( $11.25 \pm 5.03$ ) for males. Although the normal distribution was not rejected for most of data sets  
202 (Table S5), homoscedasticity was not fulfilled for some of their combinations (Table S7). Therefore,  
203 female and male data were compared within each combination of conditions and positions using Welch's  
204 t-test (Figure 3, Table S9). In alive condition (on alive female surfaces), males attached significantly better  
205 to both female surfaces than females irrespective of the position (abdomen or elytra) (Figure 3). On dead  
206 female surfaces, males attached significantly stronger to the female elytra than females, while on  
207 abdominal surfaces, females attached slightly stronger than males (Figure 3). On the contrary, no  
208 significant differences were observed between the sexes in experiments on replicated resin female surfaces  
209 (Figure 3). Among different models selected to explain the variations in the pull-off force values, the full  
210 model was found to be the best one (Table 1), meaning that all explanatory variables, i.e. sex, position and  
211 condition as well as all possible interactions among them, significantly affected the pull-off forces.  
212 However, the effects of the factor 'position' (elytra or abdomen) were negligible for all data, the male  
213 data, and the data of the condition resin replica (Table 2). The model showed that substrate conditions  
214 (dead and resin replica) positively affected pull-off forces compared to alive female surfaces, and this

215 trend was especially striking in females (Figure 3, Table 2). The predictions calculated with the full model  
216 are very close to medians in all cases and are plotted as the black dots in Figure S5.

217 Safety factors (pull-off force values divided by weights of experimental beetles) ranged from 4.19 to  
218 140.31 ( $34.81 \pm 24.13$ , mean  $\pm$  sd) in females and 25.73 to 203.01 ( $102.97 \pm 42.27$ ) in males. Although the  
219 normal distribution was not rejected for most of our data sets (Table S12), the homoscedasticity was not  
220 fulfilled for most of the combinations of them (Table S13). Then, female and male data were compared  
221 within each combination of conditions and positions using Welch's t-test (Figure 4, Table S14). In all  
222 conditions and positions, the male safety factors were significantly higher than those of the experimental  
223 females (Figure 4). The selected full model turned out to be the best to explain the variations also in the  
224 safety factors (Table 3) so that all explanatory variables (i.e. sex, position and condition) and all possible  
225 interactions among them significantly affected the safety factor values (Table 4). The predictions  
226 calculated with the full model are very close to medians in all cases and are plotted with the black dots in  
227 Figure S7.

228 The surfaces of the dead females are partially covered with debris (Figure 5) but more or less clean  
229 (Figure S8). Epicuticular micro projections are visible on the abdomen (Figure 5). On the contrary to  
230 those, the surfaces of the resin replicas are entirely covered with droplets of plausible grease on the  
231 abdomen (Figure 5, S9). The elytra surfaces of the resin replicas are in most cases clean (Figure S9) but in  
232 some cases covered with some grease droplets (Figure 5).

233

234 (c) Epicuticular grease

235 Ruptured abdomina of freshly frozen females were completely filled with the haemolymph (Figure 6a),  
236 while those of dead and dried females were hollow inside plausibly due to desiccation of the haemolymph  
237 (Figure 6g). Only on the abdominal surfaces of freshly frozen females, a grease layer covering the surface  
238 was detected (especially well seen on nanoscopic glass particles), while gaps among glass particles on  
239 dead females are clearly visible (compare Figure 6b-f with Figure 6h,i). The thicknesses of the grease  
240 layer ranged from ca. 28.0 to 62.1 nm ( $44.8 \pm 11.5$ , mean  $\pm$  sd, six positions were randomly chosen from  
241 Cryo-SEM images taken from one female).

242

## 243 4. Discussion

### 244 4.1. Effects of sexual dimorphism

245 As previously demonstrated for beetle species that male discoidal setae adhere stronger to smooth  
246 surfaces than other setal types [9-11, 14, 34, 35, 52], males of *G. viridula* adhered stronger to female  
247 surfaces than conspecific females (see Figure 3). The present study shows for the first time that the  
248 discoidal setae, which only males possess, are beneficial for males to attach them to female surfaces  
249 during mating in this beetle species. Considering that *G. viridula* shows a peculiar mating posture that a  
250 male stays on a female swollen abdomen instead of the elytra [47] and that the positions' effect (on elytra  
251 or abdomen) was negligible in the experiments performed here (Table 2), the function of the male  
252 discoidal setae previously confirmed for *C. americana* using a similar but dynamic experimental design  
253 [14] and for *G. viridula* on dead female elytra [9] seems to be universal at least for representatives of this  
254 subfamily, but probably it is also the case for many other species of the beetles from the families of  
255 Chrysomelidae and Coccinellidae having females with smooth body surfaces.

256 Another important result we obtained is markedly high safety factors in males and relatively low safety  
257 factors in females on all tested surfaces (means: ca. 15 for females and ca. 100 for males on alive female  
258 surfaces, see Figure 4) compared to those in *C. americana* measured on alive females (ca. 25 and 45 for  
259 females and males, respectively) [14]. Safety factors have been already reported previously for females  
260 and males of *G. viridula* (ca. 35 and ca. 45, respectively [33], ca. 67 for females and ca. 153 for males  
261 [42]) and another well-studied leaf beetle *Leptinotarsa decemlineata* (ca. 5 for females and ca. 15 for  
262 males [33], ca. 13 for females and 63 for males [42]). However, they were obtained on flat hydrophilic  
263 glass surfaces using traction force experiments [33] or on flat polished plexiglass surfaces using a  
264 centrifugal force device in a tangential direction [42]. Considering that adhesive forces are half of shear  
265 forces measured in the same species in general [53, 54], especially the extremely high male safety factors  
266 obtained here from *G. viridula* on alive female surfaces are surprising. The possible explanation for the  
267 high safety factor in males is the substrate curvature, since the number of tarsomeres getting in contact  
268 with substrates depends on substrate curvature [55]. Also, stronger grip forces of insects on rods compared  
269 to those on flat glasses were reported [56, 57]. Since the contribution of the discoidal setae to the  
270 attachment ability on smooth surfaces is higher than those of pointed and spatula-shaped ones, males of *G.*  
271 *viridula* conceivably use all their tarsomeres to adhere to female curved surfaces, and this resulted in the  
272 stronger male attachment on female surfaces compared to experimental females. In contrast, the female  
273 low safety factor may be attributed to the remarkably low proportion of lanceolate setae in *G. viridula*  
274 compared to *L. decemlineata* [42]. These setae are much wider than the pointed ones [42] and  
275 superficially resemble the discoidal ones. The lanceolate setae may function partially like the discoidal  
276 ones, when the latter is absent, and this may explain the relatively lower safety factor in *G. viridula*  
277 females in comparison to *C. americana* females, although the number of the lanceolate setae has to be  
278 counted in *C. americana* as well to test this hypothesis.

279 The very high attachment ability of the *G. viridula* male was already pointed out in comparative studies  
280 with *L. decemlineata* although the latter species is larger [33, 42]. The tenent setae of *G. viridula* are  
281 thinner than those of *L. decemlineata*, which provides *G. viridula* with higher attachment ability [42]. As  
282 indicated by Voigt et al. [42], the higher attachment ability is attributed to adaptations to various host  
283 surfaces for females. Especially sexually mature females in *G. viridula* cannot fly due to a remarkable egg  
284 load, and for them, attachment to host plants must be more crucial than for males. These beetles feed on  
285 various Polygonaceae plants with a particular focus on the genus *Rumex* [47]. One of the most used host  
286 plants in our study site, *Rumex obtusifolius*, whose leaf surface is characterised by cell concavities and  
287 irregularities, a coverage with a few epicuticular wax crystals [26, 42]. To be able to adhere to variously  
288 rough surfaces, females need compliant pointed and spatula-like setae.

289 For males, mating is a more crucial situation. During mating, females crawl around and go through  
290 small food marks, and thereby males are sometimes thrown off from females [47]. In addition, males are  
291 known to interrupt other males copulating, i.e. direct male-male competition, which reduces the fitness of  
292 mating males [58]. Therefore, males of *G. viridula* need to be able to adhere well to female smooth  
293 surfaces. Males also have to adhere to their host plants in order to feed on them, but slipping off from host  
294 plants is not as detrimental as in females, since males can fly a short distance. At least freshly emerged  
295 beetles can fly [47]. Moreover, it has been previously reported that the attachment ability of the discoidal  
296 setae increases just after they walk on wet surfaces [37], and the humidity directly on leaf surfaces is  
297 higher than the ambient humidity due to water vapour produced by leaves [59]. Therefore, the presence of  
298 the discoidal setae in the context of locomotion on host plants in this species might not be as unfavourable  
299 as previously thought. The division of labour between the sexes may have caused the remarkable sexual  
300 dimorphism reflected by the diverged safety factors generated on alive female surfaces.



## 302 4.2. Effects of the grease layer and substrate stiffness

303 Our results showed that substrate conditions, such as dead and replicated females, have positive effects  
304 on pull-off forces in comparison to alive female surfaces, and this trend was striking in the female  
305 attachment (see Table 2). The attachment ability tended to increase on dead and replicated female surfaces  
306 in both sexes, which indicates that the grease layer decreases the attachment. The possible explanation for  
307 this trend can be a difference in Young's moduli of tenant setae. The tips of discoidal setae are stiffer than  
308 those of pointed and spatula-shaped setae [10, 45]. Since the tarsal secretion and epicuticular grease layer  
309 have been reported to be chemically similar, these liquid layers are likely connected when the tarsi come  
310 into contact with female surfaces [18-20]. The rigid discoidal setae compared with the softer pointed and  
311 spatula-shaped setae might be more effectively and closely placed to the female body surfaces in the  
312 united grease layer, resulting in a 'good' contact of discoidal setae regardless of the presence or absence of  
313 the grease layer. Contrary, the softer pointed and spatula-shaped setae may take longer to get into contact  
314 with the female surfaces, resulting in a kind of hydroplaning and therefore 'worse' attachment. However,  
315 alive females and both dead and replicated females used for the attachment force measurements differed  
316 not only in the presence or absence of the grease layer, but also likely in surface chemistry including  
317 debris and plausibly grease droplets observed on the test surfaces (Figure 5) and stiffness due to a drying  
318 process and rather resin material used for replicas.

319 Indeed, the pull-off forces generated on dead and replicated females showed disparate trends between  
320 the sexes (Figure 3). Especially the plausible grease droplets observed on the abdominal surfaces of the  
321 resin replicas, which was not our intention, look the main cause for the discrepancy between the dead  
322 female and resin replicas at a glance. However, the pull-off forces on the abdominal and elytral surfaces of  
323 the resin replicas are very similar, indicating that the effect of the grease droplets on our experiments is  
324 negligible. Considering that the decrease of attachment ability in *G. viridula* and *L. decemlineata* has been  
325 observed on substrates having stiffness lower than 5 MPa and a flat surface [33], Young's moduli of dried  
326 elytra [60-63] and cured resin [64] are in the range a few GPs, and hence, stiffnesses of the substrates  
327 unlikely played a role in our experiments. Contrary to the case of the elytra, females tended to adhere  
328 stronger than males to the abdomen of the dead females, although the difference was statistically marginal  
329 (Figure 3). The membranous abdominal sternites were completely dehydrated, and dehydration is known  
330 to increase Young's moduli, for instance, observed in tenant setae [65]. However, the body cavity of dried  
331 females was found to be hollow inside (see Figure 6g). No visible rupture of dried sternites was observed  
332 during pull-off experiments, but the dried sternites are likely more compliant compared to the swollen  
333 abdomen filled with haemolymph and eggs. Therefore, the softer pointed and spatula-like setae may come  
334 more efficiently into contact with the dried sternites, resulting in high attachment in experimental females.

335 This explanation attributing the setal stiffness to the observed difference in attachment ability between  
336 the sexes remains a hypothesis, and a comparative experimental study on curved surfaces made of the  
337 same materials either with or without a grease layer should be conducted to prove the hypothesis. In  
338 addition, the physical property characterisation of the female sternite is also required.

## 339 5. Conclusion

340 The present study clearly showed that in *G. viridula*, the discoidal setae enable the males to adhere to  
341 female surfaces regardless of position on the female body, and it appears that the function of the discoidal  
342 setae for mating is universal in male beetles from the families Chrysomelidae and Coccinellidae having  
343 females with the smooth elytra and prothorax. Other types of tenant setae, e.g. pointed, lanceolate, and  
344 spatula-like ones, show much lower attachment ability on female surfaces presumably because of the  
345 presence of the grease layer, implying that the evolution of the male discoidal setae could be driven by a  
346 counter-adaption to the female grease layer. The divergent performance found between the sexes differing

347 in their setal composition is likely caused by the stiffness difference between the setae types and by the  
348 specific shape of the setal tips. The diverged functional specialisation of attachment pads between the  
349 sexes was confirmed. The swollen female abdomen and their peculiar mating posture in *G. viridula* are  
350 likely attributed to this remarkable divergence of labour in their attachment pads between the sexes.

351

## 352 **Acknowledgements**

353 We greatly appreciate the kind introduction to the Cryo-SEM method by Esther Appel (Kiel University,  
354 Germany) to YM and the support for setting up the attachment force measurement experiment by Clemens  
355 Schaber, Hamed Rajabi and Emre Kizilkan (Kiel University, Germany). We are grateful also for the  
356 support of the Electron Microscope Laboratory, Research Faculty of Agriculture, Hokkaido University.

357

358 **Data accessibility.** Supporting data are available in the electronic supplementary material.

359

360 **Competing interests.** We declare we have no competing interests.

361

362 **Ethics approval.** All animals were handled following the ASAB guidelines for the treatment of animals.

363

## 364 **Authors' contributions**

365 SNG and EG conceptualised the study and designed the attachment force experiment, YM carried out  
366 experiments and performed statistics, all co-authors interpreted results, YM wrote the first draft, and SNG  
367 and EG revised it. All authors contributed to and approved the final draft of the manuscript.

368

## 369 **Funding**

370 This study was partially supported by the German Research Foundation (DFG grants no. MA 7400/1-1)  
371 to YM.

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374 **Table 1.** Results of the model selection based on the Akaike information criterion. Pull-off forces are used  
 375 as response variables. All data are included. N = 144.

| Included parameters   | AIC estimation |
|---|----------------|
| sex + condition + position  | 825.01         |
| sex + condition + position + sex × condition  | 805.12         |
| sex + condition + position + sex × position   | 819.92         |
| sex + condition + position + condition × position   | 806.02         |
| sex + condition + position + sex × condition + sex × position   | 799.40         |
| sex + condition + position + sex × condition + condition × position   | 783.31         |
| sex + condition + position + sex × position + condition × position  | 800.35         |
| sex + condition + position + sex × condition + sex × position +<br>condition × position   | 776.72         |
| sex + condition + position + sex × condition + sex × position +<br>condition × position + sex × condition × position (full model) | 753.82         |

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379 **Table 2.** A linear mixed model used to analyse pull-off force data obtained. All measured data are  
 380 included. Individuals are included with a random factor. N = 144.

|   | Estimate | Std.<br>Error | df    | t value | Pr(> t )                 |
|---|----------|---------------|-------|---------|--------------------------|
| (intercept)   | 3.85     | 1.15          | 62.1  | 3.34    | <b>0.0014</b> **         |
| sex (male)  | 7.03     | 1.62          | 62.1  | 4.31    | <b>5.86e-05</b><br>***   |
| condition (dead)  | 13.65    | 1.18          | 110.0 | 11.56   | <b>&lt; 2e-16</b><br>*** |
| condition (resin replica)                                 | 4.61     | 1.18          | 110.0 | 3.90    | <b>0.00017</b><br>***    |
| position (elytra)   | 1.33     | 1.18          | 110.0 | 1.12    | 0.27                     |
| sex (male): condition (dead)                              | -11.09   | 1.67          | 110.0 | -6.64   | <b>1.23e-09</b><br>***   |
| sex (male): condition (resin replica )                    | -7.98    | 1.67          | 110.0 | -4.78   | <b>5.55e-06</b><br>***   |
| sex (male): position (elytra)                             | -2.54    | 1.67          | 110.0 | -1.52   | 0.14                     |
| condition (dead): position (elytra)                       | -8.84    | 1.67          | 110.0 | -5.29   | <b>6.22e-07</b><br>***   |
| condition (resin replica ): position (elytra)             | 0.51     | 1.67          | 110.0 | 0.31    | 0.76                     |
| sex (male): condition (dead): position (elytra)           | 10.96    | 2.36          | 110.0 | 4.64    | <b>9.72e-06</b><br>***   |
| sex (male): condition (resin replica ): position (elytra) | 4.85     | 2.36          | 110.0 | 2.05    | <b>0.043</b> *           |

381 Statistical significances are indicated with bold letters: \* > 0.05, \*\* > 0.01, \*\*\* > 0.001.

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389 **Table 3.** Results of the model selection based on the Akaike information criterion. Safety factors are used  
 390 as response variables. All data were included. N = 144.

| Included parameters   | AIC estimation |
|---|----------------|
| sex + condition + position  | 1341.83        |
| sex + condition + position + sex × condition  | 1325.63        |
| sex + condition + position + sex × position   | 1332.43        |
| sex + condition + position + condition × position   | 1319.26        |
| sex + condition + position + sex × condition + sex × position   | 1315.97        |
| sex + condition + position + sex × condition + condition × position   | 1302.36        |
| sex + condition + position + sex × position + condition × position  | 1309.34        |
| sex + condition + position + sex × condition + sex × position +<br>condition × position   | 1292.12        |
| sex + condition + position + sex × condition + sex × position +<br>condition × position + sex × condition × position (full model) | 1268.33        |

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393 **Table 4.** Linear mixed model for safety factors. Individuals are included with a random factor. All  
 394 measurements are included. N = 144.

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|   | Estimate | Std. Error | df      | t value | Pr(> t )               |
|---|----------|------------|---------|---------|------------------------|
| (Intercept)   | 13.72    | 8.49       | 53.7    | 1.62    | 0.11                   |
| sex (male)  | 85.02    | 12.00      | 53.7    | 7.08    | <b>3.09e-09</b><br>*** |
| condition (dead)  | 52.93    | 8.14       | 110.0   | 6.50    | <b>2.40e-09</b><br>*** |
| condition (resin replica )                                | 18.18    | 8.14       | 110.0   | 2.23    | <b>0.028</b> *         |
| position (elytra)   | 5.57     | 8.14       | 110.0   | 0.68    | 0.46                   |
| sex (male): condition (dead)                              | -28.58   | 11.51      | 110.0   | -2.48   | <b>0.015</b> *         |
| sex (male): condition (resin replica )                    | -49.02   | 11.51      | 110.0   | -4.26   | <b>4.35e-05</b><br>*** |
| sex (male): position (elytra)                             | -16.87   | 11.51      | 110.0   | -1.47   | 0.16                   |
| condition (dead): position (elytra)                       | -33.53   | 11.51      | 110.0   | -2.91   | <b>0.0043</b> **       |
| condition (resin replica ): position (elytra)             | 1.10     | 11.51      | 110.0   | 0.096   | 0.93                   |
| sex (male): condition (dead): position (elytra)           | 53.87    | 16.28      | 110.0   | 3.31    | <b>0.0013</b> **       |
| sex (male): condition (resin replica ): position (elytra) | 50.84    | 16.28      | 110.000 | 3.12    | <b>0.0023</b> **       |

396 Statistical significances are indicated with bold letters: \* > 0.05, \*\* > 0.01, \*\*\* > 0.001.

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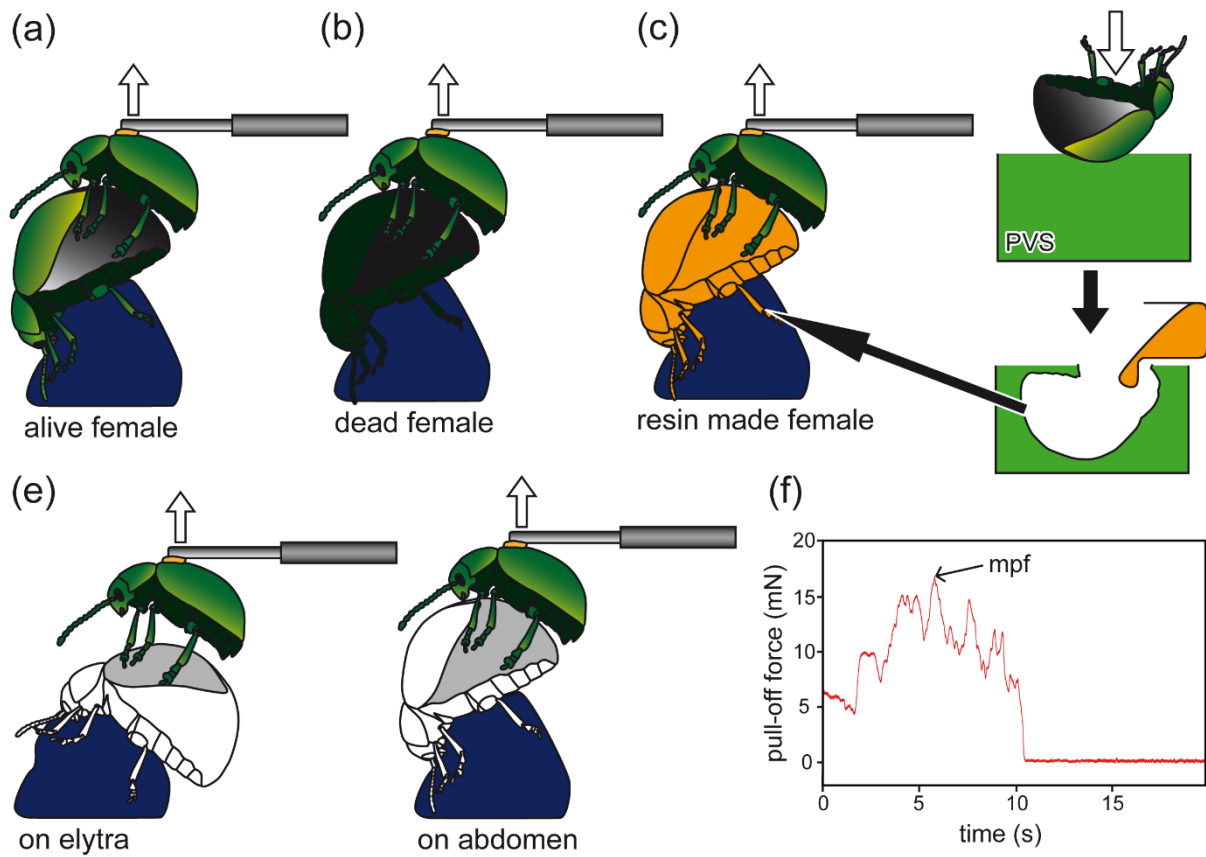
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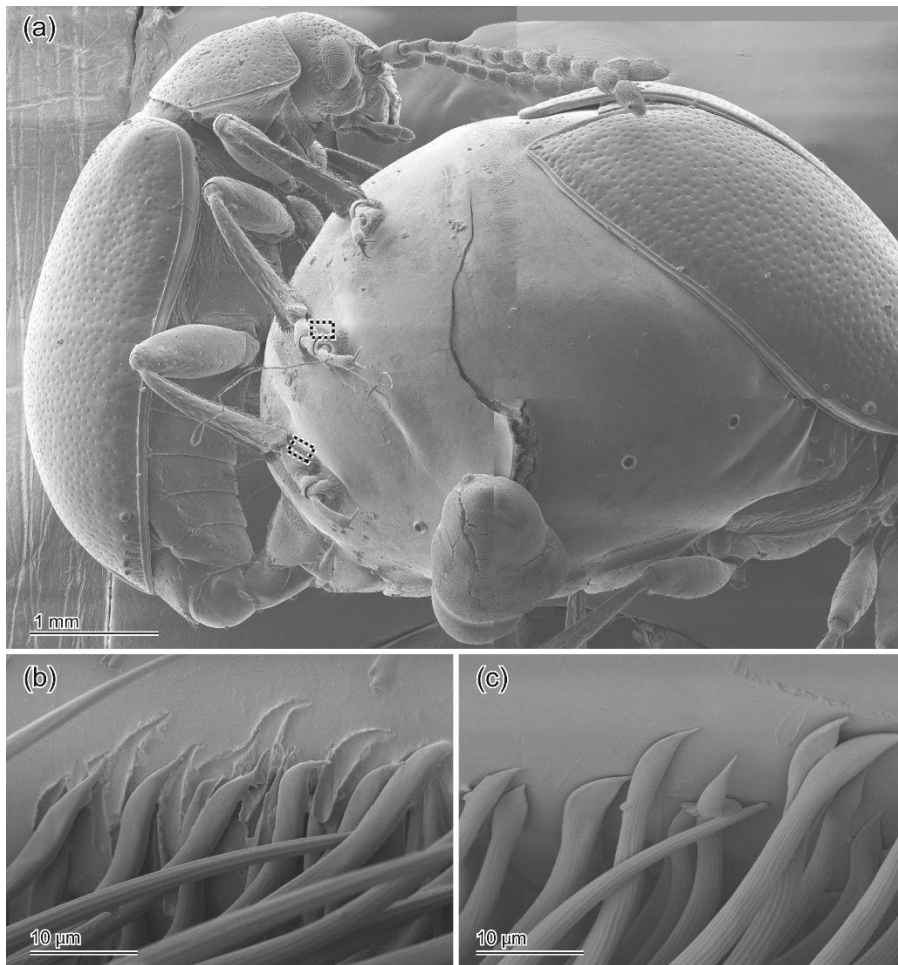
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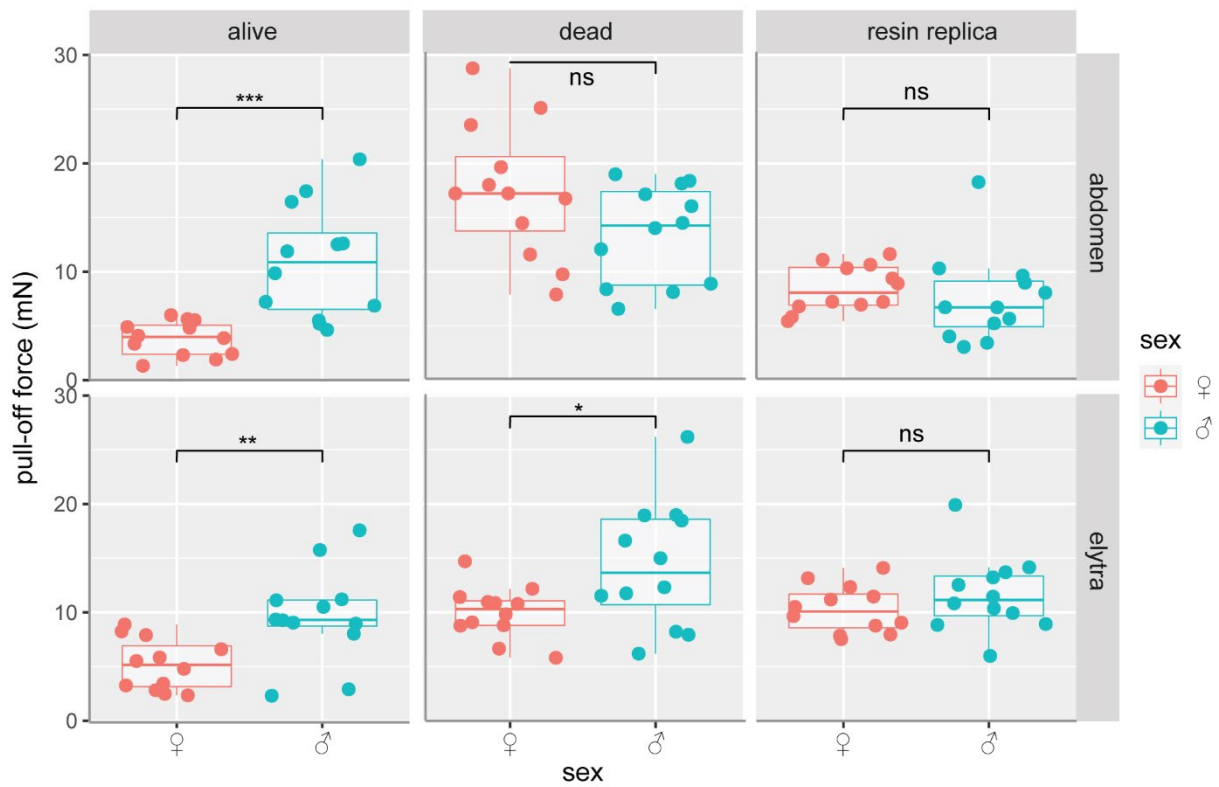
Figures



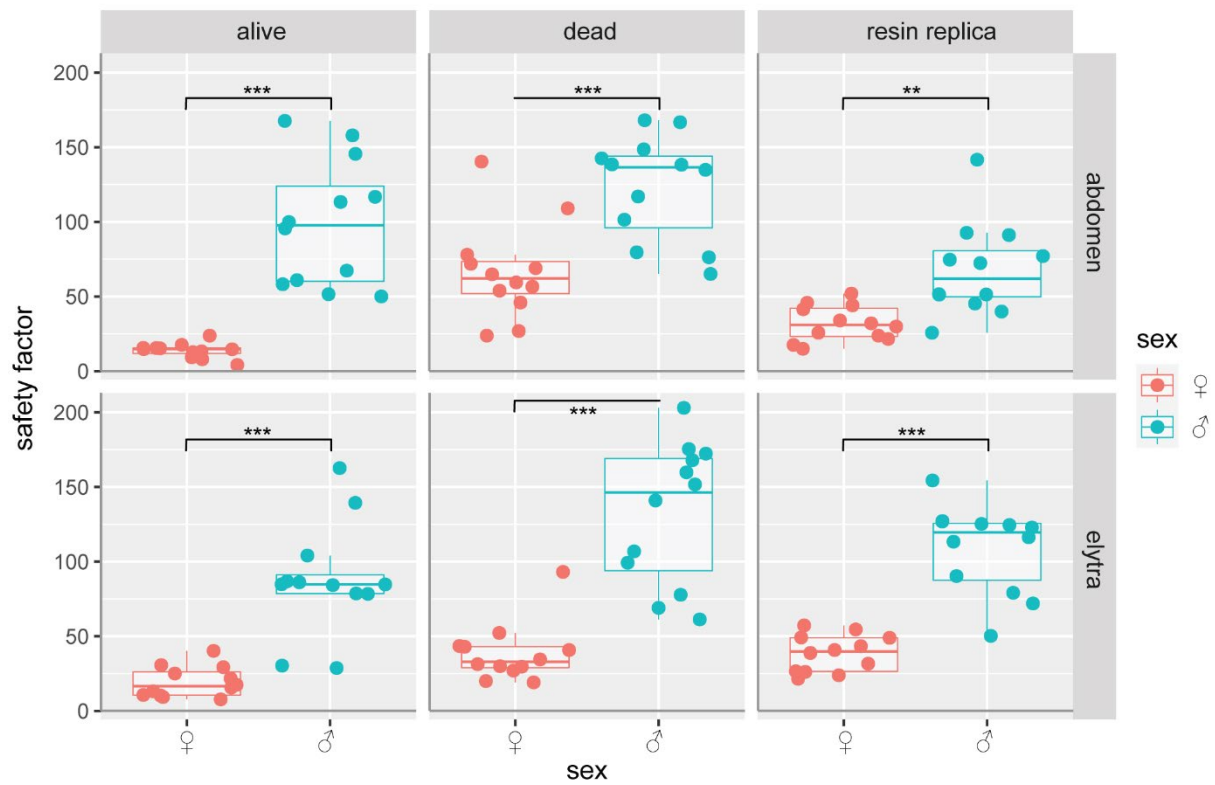
**Figure 1.** Experimental set-up for force measurements. (a-c) Tested conditions. (e,f) Tested positions. (f) An example force curve measured with a female on a dead female abdomen. mpf: maximum pull-off force; PVS: polyvinylsiloxane.



**Figure 2.** Cryo-SEM micrographs of a coupling *Gastrophysa viridula* beetles. (a) Habitus. As the female abdomen ruptured at the moment of submerging in liquid nitrogen, the frozen hemolymph is seen and highlighted with a black arrowhead. (b,c) Magnified views of the male tenent setae attached to the female abdomen (highlighted in (a)). Since the midleg moved slightly at the moment of flush freezing, setal prints are visible (highlighted with white arrowheads).



**Figure 3.** Pull-off forces generated by experimental females and males of *Gastrophysa viridula*. All measured data are included. The upper whisker (the third quartile plus 1.5 times the interquartile range), the third quartile, the median, the first quartile and the lower whisker (the first quartile minus 1.5 times the interquartile range) are shown from top to down. Obtained values are shown as points. Statistics are summarised in Table S9. N = 12 for each dataset. Statistical significances are indicated with asterisks: \* > 0.05, \*\* > 0.01, \*\*\* > 0.001.



**Figure 4.** Safety factors (pull-off force/weight) calculated for experimental females and males. All measured data are included. The upper whisker (the third quartile plus 1.5 times the interquartile range), the third quartile, the median, the first quartile and the lower whisker (the first quartile minus 1.5 times the interquartile range) are shown from top to down. Values are shown as points. Statistics are summarised in Table S14. N = 12 for each dataset. Statistical significances are indicated with asterisks: \* > 0.05, \*\* > 0.01, \*\*\* > 0.001

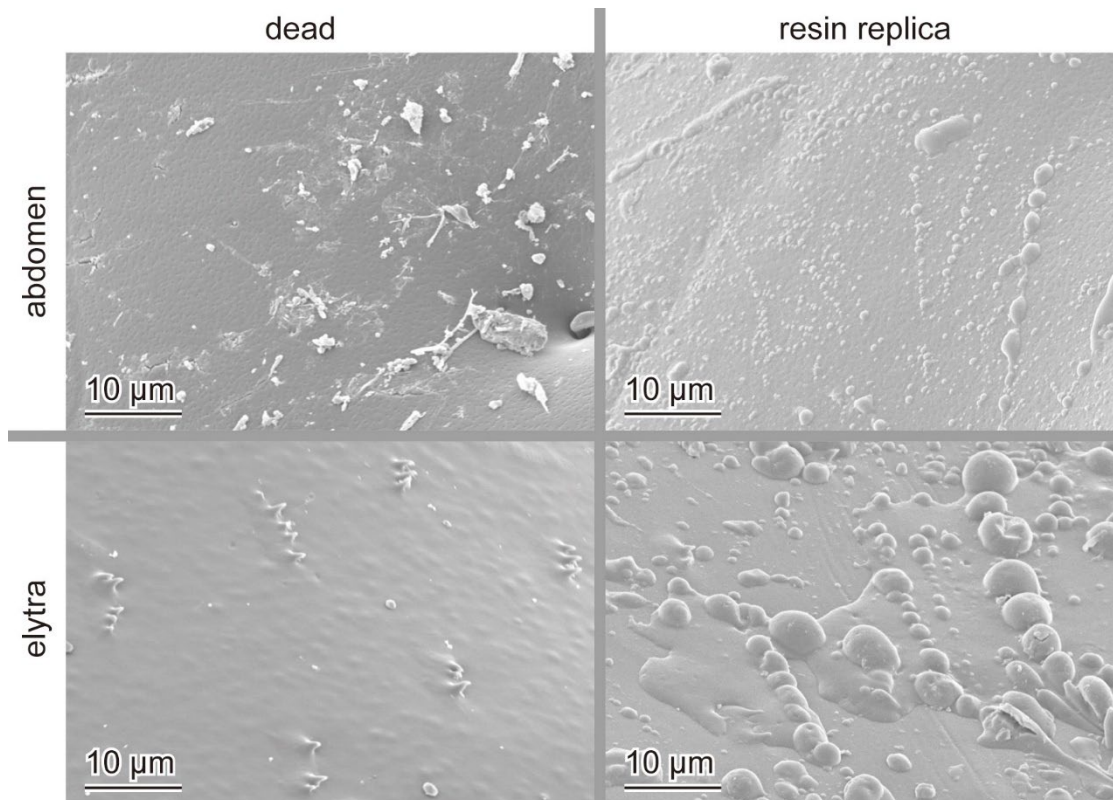
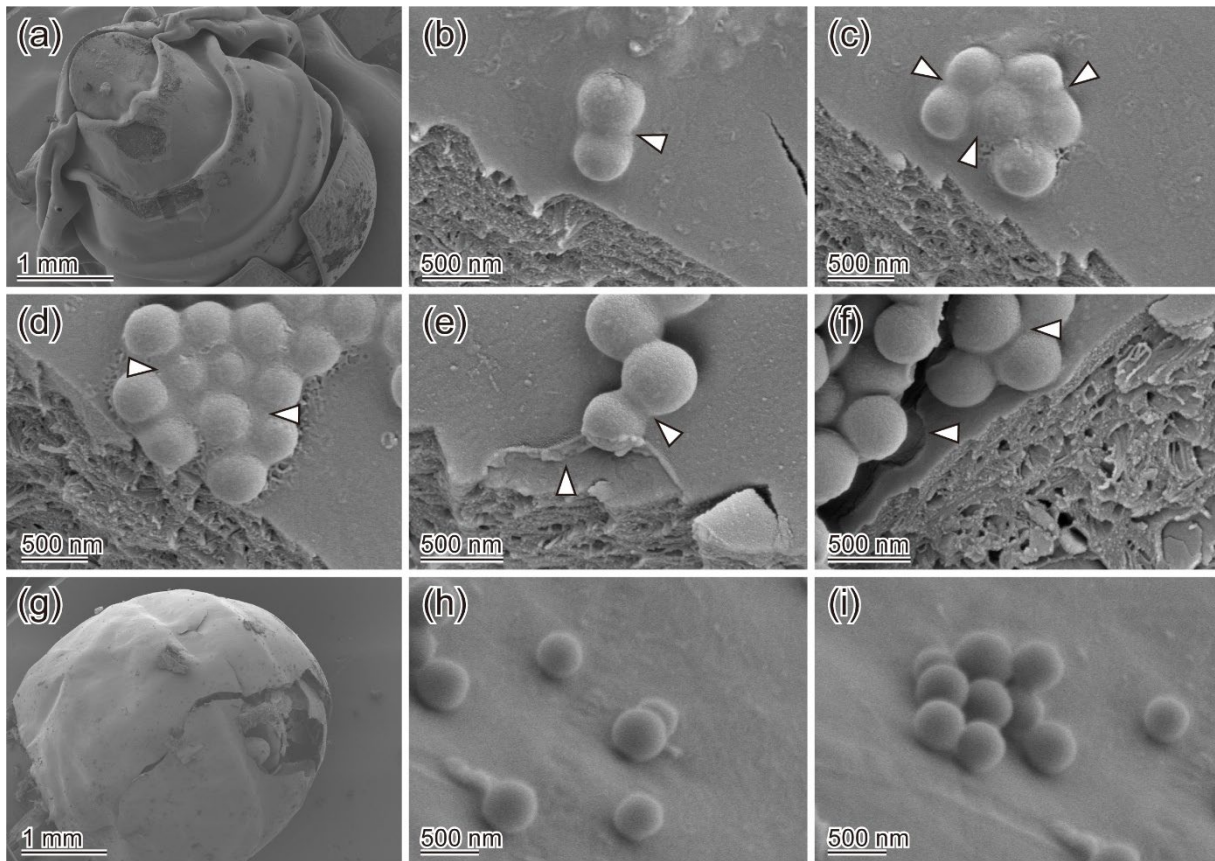


Figure 5. SEM micrographs of a dead female and a resin replica used for the force measurements.



**Figure 6.** Cryo- SEM micrographs of ruptured abdominal surfaces of *Gastrophysa viridula* females powdered with 400 nm glass particles. (a-f) Freshly frozen female used for the attachment force measurement. White arrowheads denote easily visible grease. (g-h) Dead female used for the measurement.