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Citation	Journal of the Royal Society interface, 20(205) https://doi.org/10.1098/rsif.2023.0324
Issue Date	2023-08-16
Doc URL	http://hdl.handle.net/2115/90553
Туре	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
- replicated female surfaces in both sexes, which indicates that the epicuticular grease layer on the
 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
- enables them to adhere to a wide spectrum of surfaces in nature and expand their habitats. Considering
- 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 pointed, lanceolate, and spatula-shaped setae, while males possess discoidal setae as well [4, 6]. The 35 36 evolutionary background of the origins of this sexual dimorphism has been considered that males can better adhere to female smooth and greasy elytra surfaces during copulation due to the presence of the discoidal 37 38 setae, despite the potential hindrance of this setal type on rough substrates [9-13]. Voigt et al. [14] presented the first empirical evidence supporting this hypothesis for the leaf beetle Chrysolina americana in vivo. 39 40 Another indirect evidence comes from the experimental study on the ladybird beetle Cryptolaemus montrouzieri [15]. In this species, hairy attachment structures of both females and males do not exhibit 41 sexual dimorphism and do not significantly differ in their attachment forces between the sexes. In the case 42

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].

The tarsal attachment in insects belongs to the so-called 'wet adhesion' type, since tarsal structures 46 produce an emulsive secretion [4, 16]. The entire body surface of an insect is covered with a lipid-based 47 48 liquid [12], which content is partially congruent with the tarsal secretion [8, 17-20]. Without this secretion, the attachment force decreases [21-23]. Thus, the contribution of the secretion to the tarsal adhesion is 49 50 crucial without any doubt. On the other hand, the increase of the fluid layer thickness could switch the function of the fluid from an increase of adhesive and friction forces to a lubrication. Due to this effect, the 51 52 grease layer on the female body surface could reduce tarsal adhesion [12, 24, 25]. Moreover, stiffness, chemistry, texture, and humidity of substrates are also known to influence attachment forces [7, 9, 26-37]. 53 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.

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

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

preparation chamber. Subsequently, the chamber temperature was set to -140 °C. Then, the samples were
sputter-coated in the frozen condition with gold-palladium (thickness 10 nm), transferred to the SEM main

sputter-coated in the frozen condition with gold-palladium (thickness 10 nm), transferred to the
 chamber, and examined at 3 kV acceleration.

90

91 (c) Force measurement: substrate preparation

For measuring attachment forces, 36 sexually matured females were used as either of the following three
substrates: (1) alive females, (2) dead females, and (3) resin replicas of females (Figure 1a-c). In the first

group, 12 alive females were anesthetised with CO₂ 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

anesthetised 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

101 PBS and denydrated in an ascending series of ethanol, till absolute ethanol. Subsequently, they were dried 102 using a critical point dryer (CPDA/Ouorum 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 preparated

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 experimental set-up consisting of a 100 g force sensor FORT-100 (World Precision Instruments, Sarasota, 110 FL, USA) connected to a force transducer MP 100 (Biopac Systems, Goleta, CA, USA). One day before 111 experimenting, beetles were anesthetised with CO₂, and their claws were cut off using a micro-scissor. 112 113 Since cutting the claws completely at the very basis could damage the tendon, which is connected to the unguitractor plate [47], only tip regions were removed (Figure S1). However, such claws could still hook 114 115 the elytron edge of the females used as substrates as described below (Figure 1e (left)). The 24 experimental beetles were treated as 12 sets of one female and one male for further experiments. The 116 beetles' elytra were glued to an insect pin with bee wax, and the pins were firmly fixed to the sensor tip. 117

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 μ m/sec. using a DC 3001R

120 manipulator (World Precision Instruments, USA), to which the force sensor was bound.

Prior to each set of measurements, experimental females and males were weighted using a Mettler
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

at 24.8 ± 2.4 °C and 49.0 ± 7.1 % relative humidity. To avoid a possible effect of experimental orders on

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

3.7.0 software (Biopac Systems Ltd, Goleta, CA, USA). For some sets of experiments, high-speed camerarecording 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

After the force experiments, both alive females and dead females utilised as substrates were examined
using the Cryo-SEM. To ease the visualisation of the grease thickness on these female surfaces, the

females were powdered with a tiny amount of small glass beads, dry borosilicate glass microspheres (ca.

400 nm in diameter) (Duke Scientific Corporation, Palo Alto, CA, USA) using fine tweezers.

Subsequently, the samples were instantly fixed in liquid nitrogen and immediately transferred to a cryopreparation chamber. To visualise liquids covering the surfaces, we also fractured the samples using a cold

142 preparation chamber. To visualise inquitis covering the surfaces, we also fractured the samples using a covering the surfaces, we also fractured the samples using a covering the surfaces, we also fractured the samples using a covering the surfaces, we also fractured the samples using a covering the surfaces, we also fractured the samples using a covering the surfaces, we also fractured the samples using a covering the surfaces, we also fractured the samples using a covering the surfaces, we also fractured the samples using a covering the surfaces, we also fractured the samples using a covering the surfaces, we also fractured the samples using a covering the surfaces.

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

- 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 'lmer()' function in the 'lme4' package [50]. A normal error structure and an identity link function were 152 153 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, elvtra) were incorporated. To choose the best combination of the fixed effects in terms of goodness of prediction, the Akaike information criterion 156 (AIC) was calculated for the following models: (a) models incorporating three fixed effects but no 157 158 interactions of them, (b) models incorporating three fixed effects and one of the possible combinations of interactions between two fixed effects, (c) models incorporating three fixed effects and one of possible 159 three combinations of interactions between two fixed effects, (d) models incorporating three fixed effects 160 161 and all possible three combinations of interactions between two fixed effects, and (e) model (d) plus the interaction of three fixed effects (full model). Female and male data were compared within each 162 combination of conditions and positions using Welch's t-test, after the normal distribution was tested with 163 164 the Shapiro-Wilk test and homoscedasticity was tested with the F-test. All data were plotted using the 'ggplot2' package [51]. 165

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

- posture of the study species was observed, in which males hold a female swollen abdomen with their six
- 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
- 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

All females and males used for the force measurements on experimental substrates ceased to move their legs vigorously as soon as they were placed. After a few seconds of a reassuring time, they were lifted using a micromanipulator. While being lifted, the beetles repeatedly moved all legs toward the body (Videos S1-12), which brought several peaks in force curves (Figure 1f), and the largest peak was taken as a pull-off force. In some cases, the claws hooked the elytron edge of alive or dead females; such measurements were terminated immediately after hooking the elytron edge was detected and/or a large 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
- accommodated in Tables S2-4.
- The first and the second measurements were highly correlated (Pearson correlation coefficients: 0.81 for 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
- 192 second measurements dropped largely in comparison to the corresponding first ones. The regression193 coefficient of these data without the outliers was 0.92 (0.79, 1.05), and the correlation coefficient was 0.87
- (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
- are shown below, since the obtained results were congruent (compare Tables S5, S7, S9, 2, Figure 3 with
- 199Tables S6, S8, S10, S12, Figure S6 respectively).
- 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 ± 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,
- female and male data were compared within each combination of conditions and positions using Welch's
- t-test (Figure 3, Table S9). In alive condition (on alive female surfaces), males attached significantly better
- to both female surfaces than females irrespective of the position (abdomen or elytra) (Figure 3). On dead 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
- 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
- condition as well as all possible interactions among them, significantly affected the pull-off forces.
- 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

trend was especially striking in females (Figure 3, Table 2). The predictions calculated with the full model 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 normal distribution was not rejected for most of our data sets (Table S12), the homoscedasticity was not 219 220 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 calculated with the full model are very close to medians in all cases and are plotted with the black dots in 226 227 Figure S7.

- 228 The surfaces of the dead females are partially covered with debris (Figure 5) but more or less clean
- (Figure S8). Epicuticular micro projections are visible on the abdomen (Figure 5). On the contrary to
- those, the surfaces of the resin replicas are entirely covered with droplets of plausible grease on the
- abdomen (Figure 5, S9). The elytra surfaces of the resin replicas are in most cases clean (Figure S9) but in
- some cases covered with some grease droplets (Figure 5).
- 233
- 234 (c) Epicuticular grease
- Ruptured abdomina of freshly frozen females were completely filled with the haemolymph (Figure 6a),
- while those of dead and dried females were hollow inside plausibly due to desiccation of the haemolymph(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
- 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 surfaces than other setal types [9-11, 14, 34, 35, 52], males of G. viridula adhered stronger to female 246 surfaces than conspecific females (see Figure 3). The present study shows for the first time that the 247 discoidal setae, which only males possess, are beneficial for males to attach them to female surfaces 248 during mating in this beetle species. Considering that G. viridula shows a peculiar mating posture that a 249 250 male stays on a female swollen abdomen instead of the elytra [47] and that the positions' effect (on elytra or abdomen) was negligible in the experiments performed here (Table 2), the function of the male 251 252 discoidal setae previously confirmed for C. americana using a similar but dynamic experimental design [14] and for G. viridula on dead female elytra [9] seems to be universal at least for representatives of this 253 254 subfamily, but probably it is also the case for many other species of the beetles from the families of Chrysomelidae and Coccinellidae having females with smooth body surfaces. 255

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

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 tenant setae of G. viridula are 281 thinner than those of L. decemlineata, which provides G. viridula with higher attachment ability [42]. As indicated by Voigt et al. [42], the higher attachment ability is attributed to adaptations to various host 282 283 surfaces for females. Especially sexually mature females in G. viridula cannot fly due to a remarkable egg load, and for them, attachment to host plants must be more crucial than for males. These beetles feed on 284 285 various Polygonaceae plants with a particular focus on the genus Rumex [47]. One of the most used host plants in our study site, Rumex obtusifolius, whose leaf surface is characterised by cell concavities and 286 irregularities, a coverage with a few epicuticular wax crystals [26, 42]. To be able to adhere to variously 287 288 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 surfaces. Males also have to adhere to their host plants in order to feed on them, but slipping off from host 293 294 plants is not as detrimental as in females, since males can fly a short distance. At least freshly emerged beetles can fly [47]. Moreover, it has been previously reported that the attachment ability of the discoidal 295 setae increases just after they walk on wet surfaces [37], and the humidity directly on leaf surfaces is 296 higher than the ambient humidity due to water vapour produced by leaves [59]. Therefore, the presence of 297 298 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 into contact with female surfaces [18-20]. The rigid discoidal setae compared with the softer pointed and 310 311 spatula-shaped setae might be more effectively and closely placed to the female body surfaces in the united grease layer, resulting in a 'good' contact of discoidal setae regardless of the presence or absence of 312 313 the grease layer. Contrary, the softer pointed and spatula-shaped setae may take longer to get into contact with the female surfaces, resulting in a kind of hydroplaning and therefore 'worse' attachment. However, 314 315 alive females and both dead and replicated females used for the attachment force measurements differed not only in the presence or absence of the grease layer, but also likely in surface chemistry including 316 debris and plausibly grease droplets observed on the test surfaces (Figure 5) and stiffness due to a drying 317 318 process and rather resin material used for replicas.

319 Indeed, the pull-off forces generated on dead and replicated females showed disparate trends between the sexes (Figure 3). Especially the plausible grease droplets observed on the abdominal surfaces of the 320 321 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 the resin replicas are very similar, indicating that the effect of the grease droplets on our experiments is 323 324 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 elytra [60-63] and cured resin [64] are in the range a few GPs, and hence, stiffnesses of the substrates 326 327 unlikely played a role in our experiments. Contrary to the case of the elytra, females tended to adhere stronger than males to the abdomen of the dead females, although the difference was statistically marginal 328 329 (Figure 3). The membranous abdominal sternites were completely dehydrated, and dehydration is known to increase Young's moduli, for instance, observed in tenant setae [65]. However, the body cavity of dried 330 females was found to be hollow inside (see Figure 6g). No visible rupture of dried sternites was observed 331 332 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 more efficiently into contact with the dried sternites, resulting in high attachment in experimental females. 334

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

339 5. Conclusion

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

348 349 350	specific shape of the setal tips. The diverged functional specialisation of attachment pads between the sexes was confirmed. The swollen female abdomen and their peculiar mating posture in <i>G. viridula</i> are likely attributed to this remarkable divergence of labour in their attachment pads between the sexes.
351	
352	Acknowledgements
353 354 355 356	We greatly appreciate the kind introduction to the Cryo-SEM method by Esther Appel (Kiel University, Germany) to YM and the support for setting up the attachment force measurement experiment by Clemens Schaber, Hamed Rajabi and Emre Kizilkan (Kiel University, Germany). We are grateful also for the support of the Electron Microscope Laboratory, Research Faculty of Agriculture, Hokkaido University.
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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
365 366 367	SNG and EG conceptualised the study and designed the attachment force experiment, YM carried out experiments and performed statistics, all co-authors interpreted results, YM wrote the first draft, and SNG and EG revised it. All authors contributed to and approved the final draft of the manuscript.
368	
369	Funding
370 371	This study was partially supported by the German Research Foundation (DFG grants no. MA 7400/1-1) to YM.
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373	

in their setal composition is likely caused by the stiffness difference between the setae types and by the

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

Included parameters	AIC estimation		
sex + condition + position	825.01		
sex + condition + position + sex \times condition	805.12		
sex + condition + position + sex \times position	819.92		
sex + condition + position + condition \times position	806.02		
sex + condition + position + sex \times condition + sex \times position	799.40		
sex + condition + position + sex \times condition + condition \times position	783.31		
sex + condition + position + sex \times position + condition \times position	800.35		
sex + condition + position + sex \times condition + sex \times position +	776.72		
condition × position			
sex + condition + position + sex \times condition + sex \times position +	753.82		
condition \times position + sex \times condition \times position (full model)			

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

	Estimate	Std. Error	df	t value	Pr(> t)
(intercept)	3.85	1.15	62.1	3.34	0.0014 **
sex (male)	7.03	1.62	62.1	4.31	5.86e-05 ***
condition (dead)	13.65	1.18	110.0	11.56	< 2e-16 ***
condition (resin replica)	4.61	1.18	110.0	3.90	0.00017 ***
position (elytra)	1.33	1.18	110.0	1.12	0.27
sex (male): condition (dead)	-11.09	1.67	110.0	-6.64	1.23e-09 ***
sex (male): condition (resin replica)	-7.98	1.67	110.0	-4.78	5.55e-06 ***
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	6.22e-07 ***
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	9.72e-06 ***
sex (male): condition (resin replica): position (elytra)	4.85	2.36	110.0	2.05	0.043 *
Statistical significances are indicated with bold letters: $* > 0.05$, $** > 0.01$, $*** > 0.001$.					

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

Included parameters	AIC estimation		
sex + condition + position	1341.83		
sex + condition + position + sex \times condition	1325.63		
sex + condition + position + sex \times position	1332.43		
sex + condition + position + condition \times position	1319.26		
sex + condition + position + sex \times condition + sex \times position	1315.97		
sex + condition + position + sex × condition + condition × position	1302.36		
sex + condition + position + sex \times position + condition \times position	1309.34		
sex + condition + position + sex \times condition + sex \times position +	1292.12		
condition × position			
sex + condition + position + sex \times condition + sex \times position +	1268.33		
condition \times position + sex \times condition \times position (full model)			

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

	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	3.09e-09 ***
condition (dead)	52.93	8.14	110.0	6.50	2.40e-09 ***
condition (resin replica)	18.18	8.14	110.0	2.23	0.028 *
position (elytra)	5.57	8.14	110.0	0.68	0.46
sex (male): condition (dead)	-28.58	11.51	110.0	-2.48	0.015 *
sex (male): condition (resin replica)	-49.02	11.51	110.0	-4.26	4.35e-05 ***
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	0.0043 **
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	0.0013 **
sex (male): condition (resin replica): position (elytra)	50.84	16.28	110.000	3.12	0.0023 **
Statistical significances are indicated with bold letters: $* > 0.05$, $** > 0.01$, $*** > 0.001$.					

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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 tenant 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.