

1 **Postmating sexual selection and the enigmatic jawed genitalia of**

2 ***Callosobruchus subinnotatus***

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12 **Summary**

13 Insect genitalia exhibit rapid divergent evolution. Truly extraordinary structures have
14 evolved in some groups, presumably as a result of post-mating sexual selection. To
15 increase our understanding of this phenomenon, we studied the function of one such
16 structure. The male genitalia of *Callosobruchus subinnotatus* (Coleoptera: Bruchinae)
17 contain a pair of jaw-like structures with unknown function. Here, we used phenotypic
18 engineering to ablate the teeth on these jaws. We then experimentally assessed the
19 effects of ablation of the genital jaws on mating duration, ejaculate weight, male
20 fertilization success and female fecundity, using a double-mating experimental
21 design. We predicted that copulatory wounding in females should be positively
22 related to male fertilization success. However, we found no significant correlation
23 between genital tract scarring in females and male fertilization success. Male
24 fertilization success was, however, positively related to the amount of ejaculate
25 transferred by males and negatively related to female ejaculate dumping. Ablation of
26 male genital jaws did not affect male relative fertilization success but resulted in a
27 reduction in female egg production. Our results suggest that postmating sexual
28 selection in males indeed favors these genital jaws, but not primarily through an
29 elevated relative success in sperm competition but by increasing female egg
30 production.

31

32 **Keywords:** sexual selection, *Callosobruchus*, sperm competition, sexual conflict,
33 genitalia

34

35 **Introduction**

36 Insect genitalia exhibit rapid divergent evolution (Hosken and Stockley, 2004;
37 Eberhard, 2004; Eberhard, 2010). There is now little doubt that this is due to
38 postmating sexual selection (Birkhead and Pizzari, 2002; Hosken and Stockley,
39 2004; Arnqvist, 2014), generated either by conventional cryptic female choice (CFC)
40 whereby female traits are evolving to gain benefits (Eberhard, 2006) or by sexually
41 antagonistic coevolution (SAC) whereby female traits are evolving to minimize direct
42 costs imposed by males (Arnqvist and Rowe, 2005). This coevolutionary process can
43 result in the evolution of remarkable structures, such as prominent sclerotized
44 structures of male genitalia that causes injuries to females. The function of these
45 structures have only rarely been addressed, but can involve enabling copulations
46 (Grieshop and Polak, 2012) or increasing male fertilization success by allowing
47 passage of male seminal fluid (Kamimura, 2010; Hotzy et al., 2012).

48 Seed beetles are widely employed in studies of postcopulatory sexual selection and
49 are well known for showing harmful male genital structures (Hotzy et al., 2012; Rönn
50 et al., 2007; Sakurai et al., 2012) that damage the female copulatory tract.

51 *Callosobruchus subinnotatus* (Coleoptera, Bruchinae) is a seed beetle with a
52 particularly interesting male genital morphology, as males are equipped with a pair of
53 prominent sclerotized “jaws” (Fig.1).

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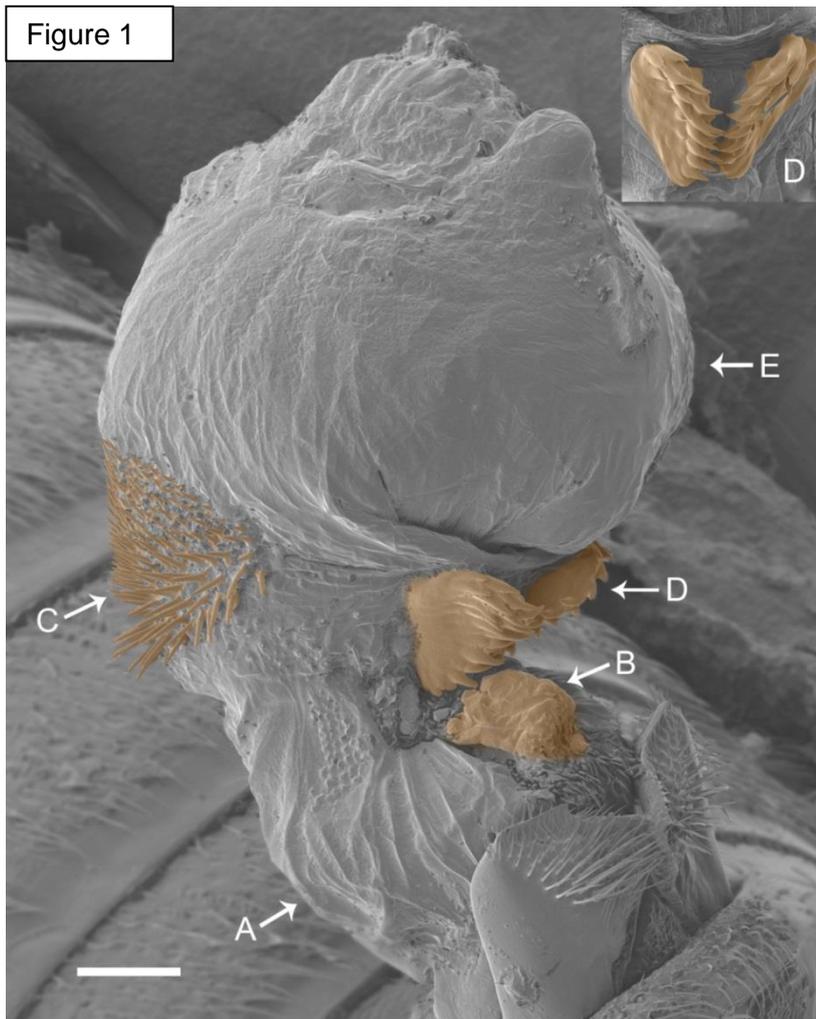
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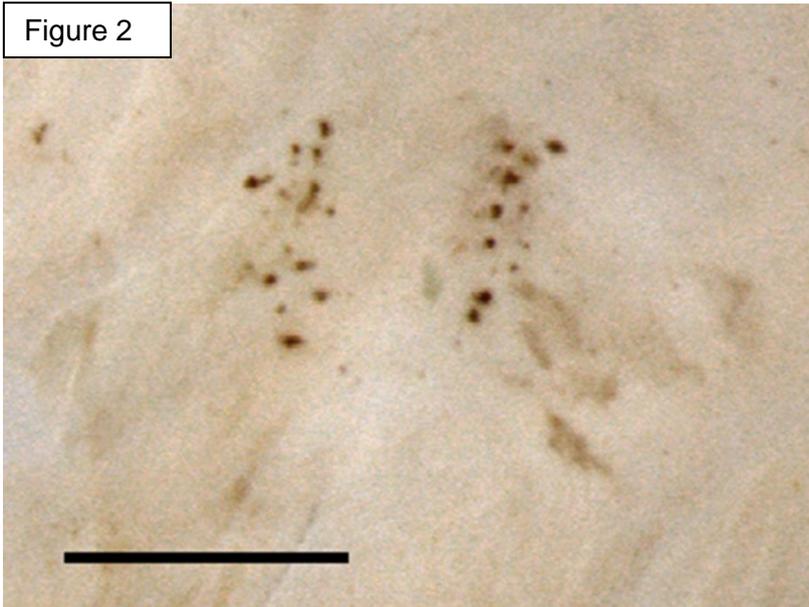
60 Fig. 1. **The remarkable male genitalia of *C. subinnotatus*.** During copulation, the genitalia unfolds which results in a
61 reformation of its armature. This starts with the expansion of the base (A) of the endophallus. On top of this base sits a
62 sclerotized structure, the basal structure (B), that appears as a thickened fold of the base of the endophallus. At this point, the
63 dorsal spines (C) are clearly visible. The jaw-like structures on the ventral side (D) then join up, due to the expansion of the
64 internal sac tip (E). At this point the jaws are closed and their position appear fixed. The endophallus is distinct from that in other
65 seed beetle species (Rönn et al. 2007). The figure shows an endophallus fixated by critical point drying to prevent tissue from
66 collapsing. Scale bar represents 100µm.

67

68 To better understand the evolution of such genetal structures, we performed a series
69 of experiments aimed at unveiling the ultimate function of these genetal jaws. The
70 jaws clearly cause injury to females: the copulatory duct is abraded or even pierced
71 by the jaws, leaving a v-shaped pattern of melanized scars (Fig. 2).

72

Figure 2



73

74 Fig. 2. The V-shaped scarring pattern caused by the jaw-like structures in the copulatory duct of females. Scale bar is
75 200µm.

76

77 We hypothesized that the genital jaws may either (1) serve as a holdfast device or (2)
78 may elevate male fertilization success by other means, as it is the case in the closely
79 related species *C. maculatus* (Hotzy and Arnqvist, 2009; Hotzy et al., 2012). Here,
80 we used phenotypic engineering to experimentally manipulate this structure. The
81 paired genital jaws bear spiny teeth-like protrusions (Fig. 1) which formed the target
82 of our manipulation: to smoothen the teeth by abrasion. A complete removal of the
83 jaws would have been interesting, but was impossible as it would have caused
84 detrimental hemorrhage.

85

86 **Materials and Methods**

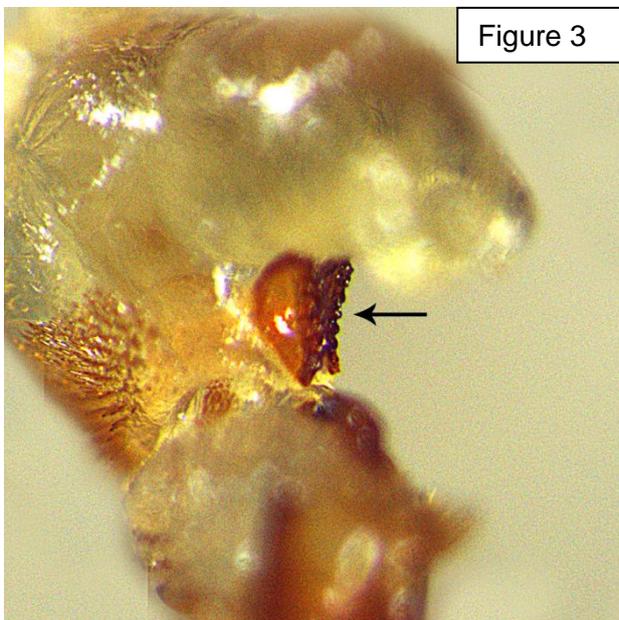
87 Beetles were mass cultured in the laboratory on a 12:12 h L:D photoperiod, 55% RH
88 and a temperature of 29°C in 1000 mL glass bottles (N=3), containing 250ml black
89 eyed-beans (*Vigna unguiculata*) per generation. New generations were set by mixing
90 beetles from each of the jars, to avoid inbreeding (Appleby and Credland, 2001). To

91 generate virgin individuals, beans with eggs were isolated individually in 24 well
92 tissue culture plates. Beetles used in the experiments described below were all of <
93 48 hours adult age and were kept individually under aphagy in aerated 5ml
94 Eppendorf tubes prior to the experiment.

95

96 *Treatment of males*

97 To assess the function of the jaw-like structures, their teeth were smoothed
98 manually following the eversion of their genitalia (Fig. 3).

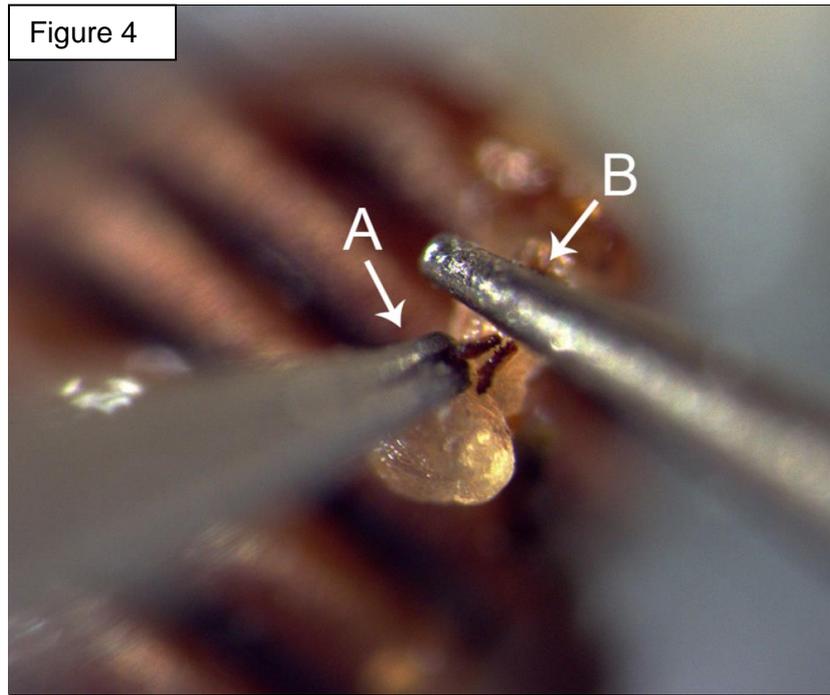


99

100 Fig. 3. **The endophallus of a *C. subinnotatus* male with smoothed teeth.** Scale bar is not available, but for references the
101 jaws are approximately 100µm.

102

103 The treatment was performed with a file made of a dentist drill (Two striper L201MF3)
104 attached to a probe. To smoothen the teeth, the jaws of lightly anesthetized (CO₂)
105 males were held in position with a forceps (SS 11200-33 Dumoxel®-Biology CE)
106 (Fig. 4) under a dissecting microscope. All male treatments (see below) were
107 performed with the same method and materials.



108

109 Fig. 4. **The manipulation of the jaws.** A male with everted endophallus is fixed with its elytra on blue-tack. The jaws are held in
110 position with a forceps (A) and then filed down using a dentist drill (B). Scale bar is not available, but for references the jaws are
111 approximately 100 μ m.

112

113 Our experimental design included four treatment groups (see Figure 5 for sample
114 sizes). [A] Some males had the teeth of their genital jaws smoothed – we refer to
115 this as the ablated jaws males (AJ). Three different control groups were created. [B]
116 One group of males were not manipulated in any way, but were left untouched (Non).
117 This group controls for potential effects of CO₂ anesthesia and genital eversion. To
118 control for ablation per se, two additional control groups were created. [C] One group
119 were treated as AJ males in every respect but instead had another structure of their
120 genitalia ablated, namely the right paramere (APa). [D] The final group of males were
121 also treated as AJ males in every respect but served as a surgical control in the
122 sense that they had a non-genitalic structure ablated, namely the rim of the pygidium
123 (APy) (Fig. S1).

124

125 Focal males were thus treated 18 hours before they were used in the experiments
126 described below. During the treatment, beetles were lightly anesthetized with CO₂ up
127 to a period of seven minutes by placing them on a FlyStuff Flypad. Virgin reference
128 males were sterilized by irradiating them with a 100 Gy dose from a cesium-137
129 source. This sterilization technique has been shown to cause lasting sterility in male
130 seed beetles while not compromising male copulation ability and sperm
131 competitive ability (Eady, 1991; Maklakov and Arnqvist, 2009). After the treatment,
132 males were placed in a 6cm petri dish with access to 5ml sugar water solution to
133 recover.

134

135 *Treatment of females*

136 Females resist mating, prior to and during copulations, by kicking males with their
137 hind legs and this might affect male fertilization success (Maklakov and Arnqvist,
138 2009). To assess the influence of female hind leg kicking on the effects of the above
139 male treatment, we also manipulated all females involved in matings with AJ and Non
140 males (not those mated to APa and APy males). One hour before the mating
141 experiment, half of the females were put on ice where the hind tibia were ablated with
142 micro scissors halfway. This renders females unable to reach, and thus resist, males
143 (Crudginton and Siva-Jothy, 2000; Edvardsson and Tregenza, 2005; Maklakov and
144 Arnqvist, 2009). To control for the effect of ablation, the other half of all females were
145 left having intact hind legs during mating but instead had their hind legs ablated one
146 hour after mating with the focal male.

147

148 *Mating experiments*

149 We measured eight different aspects of reproductive response to the genital jaw
150 manipulation: mating duration, male ejaculate weight, female ejaculate dumping, the
151 amount of scarring caused in the female copulatory duct, male offensive sperm
152 competition success, male defensive sperm competition success and female
153 fecundity. Moreover, the effect of female kicking during mating on these responses
154 was assessed.

155
156 The above reproductive responses were based on a series of standard double-
157 mating experiments in which both defense (P1) and offense (P2) components of
158 sperm competition success were measured using a standard sterile male technique
159 (Boorman and Parker, 1976; Simmons, 2001). Here, females were mated with two
160 males in succession, one of which was irradiated such that his sperm remain motile
161 and fully able to fertilize eggs but carry lethal mutations that render the eggs inviable
162 and the other male was focal and fertile. Here, P1 and P2 denote the proportion of
163 offspring that is fertilized by the focal male when he is first or second to mate,
164 respectively, with a given female in such a double mating experiment. Briefly, focal
165 experimental males, sterile reference males and females were first weighed on a
166 balance (Sartorius ME235S Genius). Mating couples were then immediately
167 introduced in pairs in 6cm petri dishes and placed in dark climate chambers under
168 rearing conditions, during very early morning which represents the peak mating time
169 *C. subinnotatus* (MBata et al., 1997). The initiation and termination of mating were
170 recorded. Pairs that did not mate within 90 minutes were discarded. After mating,
171 both male and female were weighed a second time. Females were placed individually
172 in 10cm petri dishes with +/- 40 beans and access to 5ml sugar water solution and
173 were stored in climate chambers for 48 hours. Following this intermating interval,

174 females were remated to a second male following the same protocol as for the first
175 mating. In the sperm offense assays (P2), the first male was a sterile reference male
176 and the second male was a focal experimental male. In the sperm defense assays
177 (P1), this order was reversed. The petri dish with beans and eggs from the
178 intermating interval was incubated for 10 days in a climate chamber, after which all
179 hatched and unhatched eggs were counted.

180

181 Male weight loss during mating provides a measure of male ejaculate weight in these
182 insects (Savalli and Fox, 1998; Rönn et al., 2008). The reduction in male weight
183 during copulation was significantly correlated with the increase in female weight
184 across all matings ($r = 0.39$, $P < 0.001$, $N = 326$). The fact that the correlation was not
185 stronger is primarily due to partial ejaculate dumping immediately after copulation by
186 females, a phenomenon common in seed beetles (Booksmythe et al., 2014) as well
187 as in insects in general (Perry and Rowe, 2008). In our experiments, mean male
188 weight loss was on average 18.2×10^{-5} g and mean female weight gain was on
189 average 12.7×10^{-5} g (paired t -test: $t_{325} = 8.55$, $P < 0.001$), suggesting that females
190 dump some 30% of the ejaculate on average. Here, we thus used male weight loss
191 as a measure of ejaculate weight and the difference between male weight loss and
192 female weight gain as a measure of female ejaculate dumping.

193

194 Following the second mating, the females were placed in new petri dishes provided
195 with ca. 40 black-eyed beans and a 5ml Eppendorf tube containing sugar water and
196 was allowed 7 days to lay eggs and heal copulatory injuries. After this time, females
197 were frozen (-21°C). After incubation for another ten days, the petri dishes containing
198 eggs on beans were also frozen, to prevent beetles from hatching. All eggs were

199 subsequently counted and we recorded whether each egg was hatched or
200 unhatched. Female were subsequently thawed and the copulatory duct and the bursa
201 copulatrix was separated from the female abdomen, cut open and placed on a
202 microscopic slide, enclosed in glycerin, and covered with a cover slip. The
203 dissections were performed under a Leica M165C microscope. A photo was taken of
204 the dissected bursa with a motorized Zeiss V20 with MRc5 camera and Axiovision
205 software. The images were subsequently analyzed in ImageJ. The image was
206 adjusted into an 8bit format and a threshold was set to distinguish scar tissue from
207 non-scar tissue. We quantified scarring in females as both the number of scars and
208 the area covered by scars, expressed in pixels. All scars were included, since it is not
209 possible to unambiguously distinguish between scars caused by the genital jaws and
210 other types of genital spines (Fig. 1).

211

212 ***Statistical analyses***

213 In our main models, we modelled the fertilization/reproductive success of the focal
214 male, using his mating order (first [P1] or second [P2]) as factor. For fertilization
215 success, we employed generalized linear models of the number of hatched eggs,
216 using binomial errors with a complementary log-log link function and an empirically
217 derived dispersion parameter where the total number of eggs laid after the second
218 mating was used as the binomial denominator. Conventional general linear models
219 were used for other inferences. Inferential models included our factorial variables
220 (P1/P2, male treatment, female treatment) and any covariates with noticeable effects.
221 Interactions were only included when statistically significant. Potential covariates
222 included body weight of males and females, ejaculate size, sperm dumping, mating
223 duration, scarring in females and the number of eggs laid by females between

224 matings. Models of the effects of female leg treatment were restricted to include only
225 AJ and Non males (see above). Four females that laid <4 eggs after the second
226 mating were excluded from our data set. In addition, two observations with
227 standardized residuals >4 were excluded from the analyses of scarring in females.
228 Analyses were performed with Genstat v.18 and Systat v.13.

229 **Results**

230 The overall fertilization success of the last male to mate, i.e. P2, was approximately
231 0.68 in *C. subinnotatus*. The model predictions, adjusted for covariates, were P1 =
232 0.39 (SE = 0.03) and P2 = 0.76 (SE = 0.03) but both of these values are likely
233 somewhat inflated as a result of a slight competitive advantage of normal sperm over
234 irradiated sperm.

235
236 Our inferential model of variation in male fertilization success (Table 1) was highly
237 significant overall ($F_{11,146} = 7.73$, $P < 0.001$). However, male genital treatment had no
238 significant effect on male fertilization success under sperm competition, measured as
239 the proportion of a female's offspring fertilized by the focal male, but both ejaculate
240 weights and ejaculate dumping by females was associated with male fertilization
241 success (Table 1). Interestingly, focal male fertilization success increased with his
242 ejaculate weight ($\beta' = 0.04$, $SE_{\beta} = 0.01$) and decreased with female ejaculate
243 dumping ($\beta' = -0.02$, $SE_{\beta} = 0.01$). We found no significant effect of female leg
244 treatment or any other covariates on male fertilization success.

245
246 **Table 1.** Analysis of deviance of a generalized linear model of variation in male fertilization success under sperm competition in
247 our double-mating experiment.

248

Source	DF	Deviance	Deviance ratio	<i>P</i> (from <i>F</i>)
P1/P2	1	845.83	56.08	<0.001
Male treatment	3	99.65	2.20	0.090
P1/P2 × male treatment	3	59.97	1.33	0.268
Eggs laid between matings	1	26.91	1.78	0.184
Focal male ejaculate weight	1	103.48	6.86	0.010
Reference male ejaculate weight	1	85.42	5.66	0.019
Ejaculate dumping	1	61.32	4.07	0.046
Residual	146	2202.11		

249

250 A two-way linear model of variation in mating duration showed that males mated
251 somewhat longer when mating as a female's first (24.3 min) compared to second
252 (21.2 min) mate ($F_{1,104} = 4.24$, $P = 0.042$), and that females with ablated hind-legs
253 mated for longer (24.3 vs. 21.2 min) ($F_{1,104} = 4.40$, $P = 0.038$), although male genital
254 treatment had no significant effect on mating duration ($F_{1,104} = 0.02$, $P = 0.89$). An
255 analogous model of variation in male ejaculate weight showed that larger males
256 transfer heavier ejaculates ($F_{1,103} = 6.46$, $P = 0.013$), while P1/P2, female treatment
257 and male treatment had no significant effects (all $P > 0.145$). Interestingly, a model of
258 female sperm dumping, simultaneously including both focal male body and ejaculate
259 weight, showed that females dumped more ejaculate from relatively small males ($\beta' =$
260 -0.019 , $SE_{\beta} = 0.004$; $F_{1,102} = 22.2$, $P < 0.001$) with relatively large ejaculates ($\beta' =$
261 1.29 , $SE_{\beta} = 0.09$; $F_{1,102} = 215.9$, $P < 0.001$), but showed no effects of P1/P2, female
262 treatment or male treatment (all $P > 0.082$).

263

264 A model of the number of scars in females, including the mating duration of both
265 matings, revealed that the mating duration of the reference male ($F_{1,100} = 4.51$, $P =$
266 0.036) was positively related to scarring and that females with ablated hind legs
267 suffered fewer scars on average (136.2, $SE = 7.7$) than did females with intact hind
268 legs (167.8, $SE = 8.3$) ($F_{1,100} = 7.45$, $P = 0.007$) but showed no effect of male
269 treatment ($F_{1,100} = 1.94$, $P = 0.166$), suggesting that female resistance during
270 copulation increases copulatory wounding. We failed to find any significant effects of
271 any predictors on the area of scarring in females.

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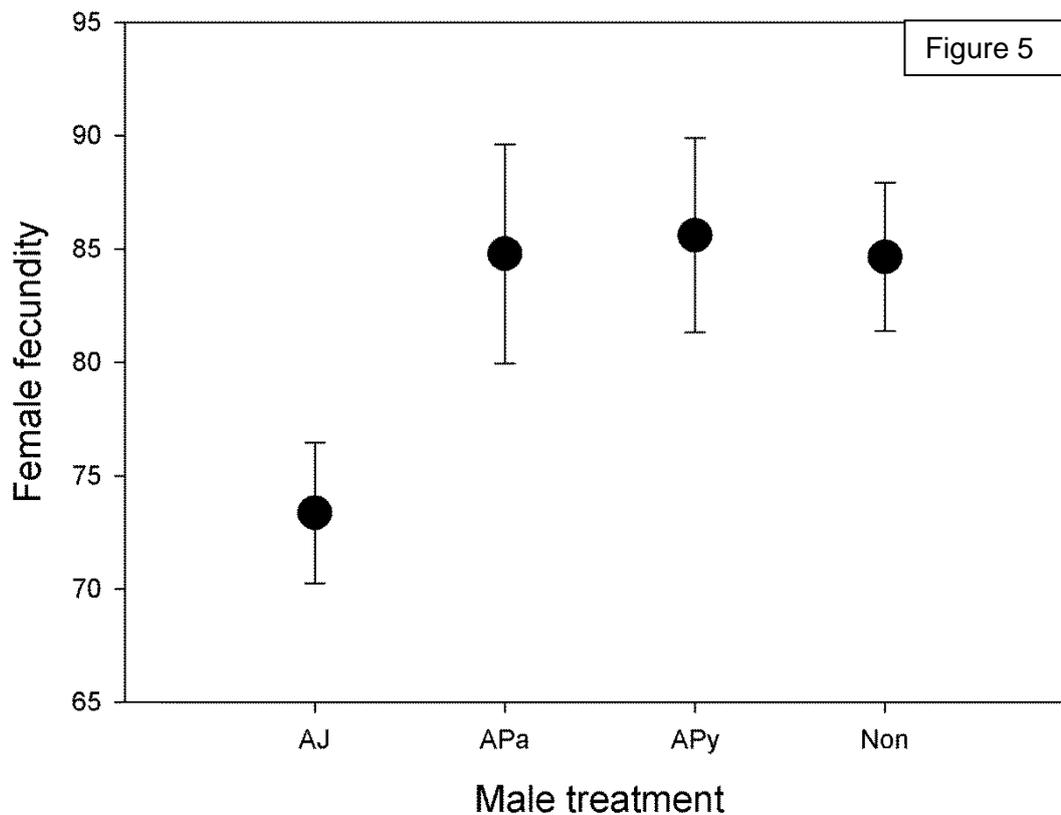
273 Female fecundity, i.e. the total number of eggs laid during our experiment, was
274 positively associated with mating duration and tended to be positively related to
275 ejaculate weight (Table 2). Notably, female fecundity was also affected by male
276 genital treatment (Table 1), such that females laid fewer eggs if her focal male mate
277 had ablated genital jaws (Fig. 5). This was true also in a reduced model, involving
278 only AJ and Non males, where male treatment had a significant effect ($F_{1,96} = 4.41$, P
279 $= 0.038$) while P1/P2, female treatment and their interaction had no significant effects
280 ($P > 0.074$ in all cases).

281

282 **Table 2.** Analysis of variance of female fecundity.

Source	DF	MS	F	P
Male treatment	3	1562.8	3.0	0.032
P1/P2	1	328.6	0.6	0.428
Focal male ejaculate weight	1	594.5	1.1	0.287
Reference male ejaculate weight	1	894.7	1.7	0.192

Source	DF	MS	F	P
Focal male mating duration	1	6.4	0.0	0.912
Reference male mating duration	1	2135.3	4.1	0.044
Residual	148	519.8		



283

284 Fig. 5. **The total number of eggs laid by females.** Females laid fewer eggs if one of her mates had ablated
285 genital jaws (AJ) (GLM: $F_{3,148} = 3.01$, $P = 0.032$. $N=55$ for AJ, $N=23$ for APa, $N=29$ for APy, and $N=50$ for Non),
286 compared to mates from the control groups. Shown is marginal mean (\pm SE) number of eggs.

287 Discussion

288 In contrast to the studies of the congener *C. maculatus* by Hotzy and Arnqvist (2009)
289 and Hotzy et al. (2012), we found no significant effects of experimental ablation of
290 genital spines on male fertilization success in *C. subinnotatus*. Instead, fertilization

291 success was determined primarily by male ejaculate weight and the degree to which
292 females dumped the ejaculate after mating. This suggests that females may affect
293 male fertilization success, by differential uptake of male seminal fluid from relatively
294 large males (Eberhard, 1996). Most importantly, we found that females laid fewer
295 eggs following mating with males with ablated genital jaws, suggesting that this
296 structure may ultimately function to stimulate female egg production more than
297 female sperm use.

298

299 Hotzy et al. (2012) found that male seminal fluid is transported across the walls of the
300 copulatory tract less rapidly in males with ablated genital spines and that such males
301 suffer reduced fertilization success as a result. Our results suggest that the genital
302 jaws of *C. subinnotatus* may also affect female uptake of male seminal fluid, although
303 this may then be manifested as elevated female egg production in this species. We
304 note that that male seminal fluid in seed beetles contains a very large number of
305 proteins, some of which affect male fertilization success and others that affect female
306 egg production (Goenaga et al., 2015; Yamane et al., 2015; Bayram et al., 2017).
307 Needless to say, given everything else equal, male postmating reproductive success
308 is elevated by an increase in female egg production (Arnqvist and Rowe, 2005).
309 Thus, our results provide support for a role of postmating sexual selection in the
310 evolution of the genital jaws in *C. subinnotatus*, although the proximate mechanism is
311 somewhat unclear and may differ somewhat from that seen in *C. maculatus* (Hotzy
312 and Arnqvist, 2009; Hotzy et al, 2012). It is interesting to note that the fact that male
313 ejaculate weight and the degree to which females dump ejaculate after mating
314 determines male fertilization success is consistent with an important role for seminal
315 fluid in mediating male postmating reproductive success also in *C. subinnotatus*.

316

317 We found that mating duration was positively associated with scarring in females, as
318 has previously documented in *C. maculatus* (Crudginton and Siva-Jothy, 2000), and
319 that females that were made unable to resist males by kicking suffered less scars.
320 This shows that the physical act of resistance by females actually acts to aggravate
321 the injuries they sustain during copulation. Ablation of genital spines decreases the
322 amount of scarring suffered by female in *C. maculatus* (Hotzy and Arnqvist, 2009;
323 Hotzy et al., 2012), but we found no significant effect of genital jaw ablation in *C.*
324 *subinnotatus*. It is possible that our ablation treatment was too subtle to generate an
325 effect on scarring in females strong enough for detection, in the face of rather
326 extensive scarring in females caused by other genital spines.

327

328 Although it is certainly possible that the enigmatic genital jaws of male *C.*
329 *subinnotatus* serves additional functions, we show here that spines on these jaws act
330 to increase female egg production rate and are hence favored by postmating sexual
331 selection. Whether sexually antagonistic coevolution has been involved in their
332 elaboration is, however, less clear since sexual conflict relies on the demonstration of
333 direct costs to females (Arnqvist and Rowe, 2005; Fricke et al., 2009). An elevation of
334 egg production rate may come at a nest cost to females (Arnqvist and Rowe 2005),
335 but this depends on how increased the rate of egg production trades-off with other
336 fitness components (Rönn et al., 2006). Similarly, our experiments suggest that the
337 increased scarring caused by the genital jaws is relatively marginal and direct costs
338 to females may thus be minor. Additional studies are required to further clarify the
339 role of the genital jaws in *C. subinnotatus* and to assess the degree to which this
340 remarkable structure is detrimental to females.

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342

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