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5 X-Ray micro-CT scanning reveals temporal separation of male harm and female

6 kicking during traumatic mating in seed beetles

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10 Liam R. Dougherty*¹ & Leigh W. Simmons¹

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16 ¹School of Biological Sciences, Centre for Evolutionary Biology, University of Western Australia,

17 Crawley, WA 6009, Australia

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19 *Email: liam.dougherty@uwa.edu.au

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22 Running head: Copulatory wounding in seed beetles

23

24 **Abstract**

25

26 In the seed beetle *Callosobruchus maculatus*, the male intromittent organ is covered in
27 sharp spines that pierce the female copulatory tract wall during mating. Though the fitness
28 consequences of traumatic mating are well studied in this species, we know much less
29 about how the male and female genitalia interact during mating. This is partly due to the
30 fact that genital interactions occur primarily inside the female, and so are difficult to
31 observe. In this study we use X-ray micro-CT scanning to examine the proximate
32 mechanisms of traumatic mating in *C. maculatus* in unprecedented detail. We show that
33 this technique can be used to identify female tissue damage before the melanisation of
34 wound sites. We visualise the positioning of the male intromittent organ inside the female
35 copulatory tract during mating, and show how this relates to tract wounding in three
36 dimensions. By scanning pairs flash-frozen at different times during mating, we show that
37 significant tract wounding occurs before the onset of female kicking. There is thus some
38 degree of temporal separation between the onset of wounding and the onset of kicking,
39 which supports recent suggestions that kicking is not an effective female counter-adaptation
40 to reduce copulatory wounding in this species. We also present evidence that the sharp
41 teeth protruding from the female tract wall are able to pierce the spermatophore as it is
42 deposited, and may thus function to aid sperm release.

43

44 **Keywords:**

45 *Callosobruchus maculatus*; copulatory wounding; genitalia; sexual conflict; traumatic
46 mating; X-Ray micro-CT.

47

48 **Introduction**

49

50 Traumatic mating (also known as copulatory wounding) is an extreme form of sexual conflict
51 observed in some animal species in which the male reproductive anatomy physically harms
52 the female during mating [1]. For example, in several insect species males possess sharp,
53 toughened spines on the intromittent organ (or aedeagus) which pierce and wound the
54 walls of the female copulatory tract during mating (e.g. [2][3][4][5]). Importantly, traumatic
55 mating may also lead to a reduction in female fitness, for example due to heightened
56 immune activity or an increased risk of infection at the site of wounding [6]. However, such
57 fitness costs have proven hard to detect, because females are expected to rapidly evolve
58 counter-adaptations to reduce any male-imposed costs [7][8]. Nevertheless, the phenotypic
59 adaptations exhibited by females are not hidden, and may be behavioural, physiological and
60 morphological in form [1].

61

62 Males of the seed beetle *Callosobruchus maculatus* are well known for their extreme genital
63 morphology. The aedeagus is covered in sharp spines which pierce and damage the walls of
64 the female copulatory tract during mating, harming the female [3]. Notably, the walls of the
65 female tract are much thicker and more highly folded in *C. maculatus* when compared to
66 closely related species in which males lack aedeagal spines [9], and a thicker tract wall (in
67 relation to aedeagal spine length) significantly reduces the degree of copulatory wounding
68 females receive [10]. Traumatic mating appears to be selected for in this species not
69 because of the harm it does to females *per se*, but because males with longer spines have
70 greater competitive fertilisation success [11]. It has been hypothesised that this effect is
71 mediated by male seminal products which can influence female reproductive physiology

72 and behaviour, and which can more effectively pass into the female haemolymph following
73 traumatic mating [12][13].

74

75 Female *C. maculatus* may also have behavioural adaptations to reduce harm during mating.

76 For example, females use their hind legs to kick males during the latter stages of mating

77 [3][14][15]. It has been shown experimentally that female kicking reduces the duration of

78 copulation [3][16], and that females able to kick sustain fewer tract wounds during mating

79 [3]. It has therefore been traditionally assumed that kicking functions to dislodge the male

80 sooner and thus reduce copulatory wounding. However, the results of two recent studies

81 challenge this view. The first found that females able to kick their mates for longer showed

82 reduced survival, and that both copulation and kicking duration increased when rival males

83 were present, suggesting that these two traits are under male control to some extent [15].

84 The second found that copulation duration was repeatable when males and females mated

85 repeatedly with the same mate (when no rivals were present), suggesting that copulation

86 duration is the product of the interaction between males and female during mating [17].

87 These results suggest that female kicking on its own may be ineffective at reducing

88 copulation duration. Further, the relationship between copulation duration and female

89 fitness is complex: longer matings can lead to greater harm to females via copulatory

90 wounding [3], but they also lead to the transfer of larger spermatophores which can directly

91 and indirectly increase female fitness [18][19]. Accordingly, longer mating durations have

92 been shown to both increase [14][18] and decrease [3][16] different measures of female

93 fitness.

94

95 Therefore, the relationship between mating duration and female fitness is complex, and the
96 function of female kicking remains unclear. If females kick to prevent copulatory wounding,
97 then we would predict that kicking should start at the onset of wounding. Alternatively, if
98 kicking exacerbates wounding then we expect to observe an increase in the rate of
99 wounding following the onset of kicking. Determining when tract wounding occurs during
100 mating may thus allow us to clarify the role of female kicking during traumatic mating. This
101 could be done by flash-freezing pairs at different times during mating, and then examining
102 the female copulatory tract for signs of wounding. However, female tract wounding in *C.*
103 *maculatus* has typically been determined by counting the area of melanised tissue formed
104 around the tract wounds several hours after mating [3][11]. Flash-freezing prevents this
105 melanisation from happening, and so we need another method of assessing tract wounding
106 if we are to use this technique. One way to solve this problem is by using X-Ray micro-
107 computed tomography (micro-CT) scanning. X-Rays easily penetrate the soft tissues of
108 insects, thus enabling the visualisation of internal structures without the destruction of the
109 sample [10]. Additionally, with appropriate staining [20] we should be able to see physical
110 signs of tissue damage in micro-CT data that are difficult to see prior to melanisation when
111 using light microscopy.

112

113 Importantly, micro-CT in conjunction with flash-freezing allows us to visualise the
114 interactions between the male and female genitalia during mating (e.g.
115 [21][22][23][24][25]), without having to destroy the sample. While *C. maculatus* has been
116 incredibly well studied in terms of the fitness consequences of mating, the interaction
117 between male and female genitalia during mating has not been studied in any detail. As a
118 consequence, our ability to identify the functional mechanisms and anatomical structures

119 involved in sexual conflict in this species is limited. For example, female *C. maculatus*
120 possess a row of chitinous teeth [26][27][28] on the inside of the copulatory tract near the
121 entrance to the bursa copulatrix (the site of spermatophore deposition). The teeth may
122 function to pierce the spermatophore and aid sperm release [14][28], as seen for example in
123 Lepidoptera [29][30]. Alternatively, they may function to limit the depth of intromission of
124 the aedeagus so that the spines do not damage the thin walls of the bursa [28]. If this is the
125 case, then it raises the possibility of the teeth physically damaging the male endophallus,
126 perhaps as another counter-adaptation to male harm. In order to distinguish between these
127 (and other) hypotheses for the function of the bursal teeth we first need to determine
128 which male structures physically contact the teeth during mating.

129

130 In this study we used contrast-enhanced X-Ray micro-CT to visualise the interactions
131 between male and female genitalia during traumatic mating in *C. maculatus*. We scanned 19
132 mating pairs of *C. maculatus* following flash-freezing in liquid Nitrogen. This allowed us to
133 visualise the positioning of the male aedeagus inside the female copulatory tract, and
134 examine how this positioning varies across pairs. We looked for evidence of an interaction
135 between the female bursal teeth and either the male aedeagus or spermatophore. We also
136 looked for signs of female tract wounding before the onset of melanisation, either in the
137 form of aedeagal spines embedded in the walls of the tract (e.g. [23]), or in the form of
138 holes and tears in the tract wall lining. By scanning pairs frozen at different time-points
139 during mating we were also able to determine whether there are significant changes in the
140 position of the aedeagus over the course of mating, and when tract wounding occurs in
141 relation to mating duration and the onset of kicking.

142

143 **Methods**

144

145 *Experimental design and sample preparation*

146 Beetles were raised on mung beans under constant conditions at 30 ± 0.5 °C and $60 \pm 10\%$

147 RH with a 12:12 h L:D cycle. On emergence, virgin adults were separated into Eppendorf

148 tubes with 2-3 other same-sex individuals. Matings were performed 1-4 days after adult

149 emergence. A single male and female were introduced into a 0.5mL Eppendorf tube and

150 allowed to mate. In order to preserve the positions of the male and female genitalia in

151 copula, the pair was flash-frozen by plunging the tube into liquid Nitrogen for 10 seconds.

152 Pairs were frozen at one of four time points: 1) after one minute (no kicking), 2) after five

153 minutes (no kicking), 3) after 30 seconds of kicking, and 4) after two minutes of kicking.

154

155 Following freezing, each pair was immediately placed into a 4% paraformaldehyde solution

156 overnight in order to fix the tissues. Samples were then stained in order enhance the X-ray

157 attenuation and increase contrast in soft tissues [20]. Samples were first dehydrated using a

158 graded series of ethanol solutions (25%, 50%, 75% and 100% x2) for one hour each. Samples

159 were then placed in a solution of 1% iodine in 100% ethanol (I2E: [20]) for 24 hours. After

160 staining, samples were stored in 100% ethanol at room temperature and scanned within 1

161 to 4 months. Each pair was stained whole (i.e. no body parts were removed) so as not to risk

162 disturbing the positioning of the male and female reproductive organs. As a result, the

163 penetration of the stain through the female abdomen and the resulting contrast of the

164 female tissue was quite poor. Nevertheless, manual segmentation of male and female

165 tissues was still possible.

166

167 *X-Ray micro-CT scanning*

168 Nineteen mating pairs were scanned in total. Pairs were scanned using a Zeiss Versa 520 X-
169 Ray microscope located at the University of Western Australia's Centre for Microscopy,
170 Characterisation and Analysis (CMCA). Pairs were scanned in 100% ethanol to prevent the
171 tissues from drying out during the scan. We focused the scans on the posterior region of the
172 female abdomen. We used the same machine settings for all pairs. The source voltage and
173 power was set at 40kV and 3W, respectively. The source and detector were placed in the
174 same position relative to the sample mount, using the 4X lens, resulting in a voxel size of
175 1.45 μm for all scans. Scans were run for 3201 projections through 360 degrees with a 10
176 second exposure for each projection, giving a total scan time of approximately 10.25 hours
177 per sample. A camera binning of 2x was used to achieve a suitable signal to noise ratio,
178 resulting in 1010 x 1010 pixels per image. No filter was used when collecting images.
179 Secondary references were collected using the LE2 filter. Scan data was reconstructed using
180 the Zeiss reconstructor package (v10.6.2005, Zeiss). Prior to reconstruction, a standard
181 centre shift and beam hardening correction was made, the default recon filter was set to
182 smooth (kernel size = 0.7) and no ring removal was applied.

183

184 *Data analysis*

185 The micro-CT data was analysed in two and three dimensions using Avizo 6 (FEI software).
186 Differences in the form and positioning of different male and female structures (and their
187 interactions) were assessed by viewing the raw slice images. We detected two signs of
188 copulatory wounding (Figure S1): aedeagus spines embedded in the female tract at the time
189 of freezing (which we refer to as 'penetrating spines'), and holes in the female tract from
190 earlier penetrations. Importantly, these holes are not seen in CT-scans of unmated females

191 (L. Dougherty, pers. obs.), and so we are confident that they reflect tract wounds and not
192 flash-freezing artefacts. For each female we counted the number of holes and penetrating
193 spines across the entire length of the tract. As the distinction between holes situated close
194 together was not always unambiguous, we took an average of two counts (blind to each
195 other). We also determined the total volume (size) of all holes by selecting all pixels inside
196 the lumen of each hole, and converting the number of pixels into μm^3 . Again for each pair
197 we did this twice and used the average total volume for analysis.

198

199 We produced 3D visualisations of representative male and female structures in order to aid
200 interpretation. We did this in two ways. First, we ‘manually dissected’ specific male and
201 female structures from inside the female abdomen during mating. To do this, the specific
202 anatomical structures of interest were first manually selected on a slice-by-slice basis using
203 the paintbrush tool, and then assigned all selected voxels to a designated material. The
204 paintbrush tool was used in conjunction with the threshold tool to only select those voxels
205 that corresponded to male or female tissue (i.e. were relatively bright). We then used the
206 mask tool to create a new dataset containing only those voxels assigned to a material.
207 Second, we also produced a 3D visualisation of an entire female abdomen in cross-section in
208 order to visualise the size of the spermatophore as it is being transferred. To do this, we first
209 used the threshold tool to select all voxels corresponding to male or female tissue (i.e.
210 above a specific brightness threshold), across all slices, which were then assigned to a new
211 material. The ‘remove islands’ tool was then used in order to remove noise due to low tissue
212 contrast. This has the effect of removing small, isolated voxels from the assigned material,
213 and so makes the larger tissue structure easier to see. In both cases we used the ‘volume
214 rendering’ tool to visualise the assigned voxels across all slices in three dimensions. For all

215 volume rendering cubic interpolation was used to smooth the volume surface, and pre-
216 integration was used to remove slicing effects. We also used the animation editor to create
217 videos showing the aedeagus of two males in three dimensions, the female copulatory tract
218 before and after the addition of a virtual slice. These videos have been archived at Dryad
219 (DOI: <http://dx.doi.org/10.5061/dryad.33243>).

220

221 All statistical analysis was performed in R v3.2.2 [31]. We tested whether the number or
222 volume of holes in the female tract was influenced by experimental treatment, using
223 analysis of variance. We visualised the radial location of tract wounding in relation to the
224 aedeagal spines by first visualising the aedeagus, female bursal teeth and holes in the
225 female tract in three dimensions using the method described above. We then rotated the
226 entire 3D volume to the same orientation (looking anteriorly, aedeagus in the centre and
227 the bursal teeth at 0°), and took a screenshot. We then drew a line from each tract hole and
228 the centre of the aedeagus, and measured the angle of each line from 0°, using the software
229 package ImageJ v1.50i [32]. From this we created circular plots using the R package
230 “circular” v0.4-7 [33] showing the direction of tract wounds in relation to the centre of the
231 male aedeagus across all females. Note that this method assumes that there is little
232 rotational movement of the aedeagus during mating.

233

234 **Results and discussion**

235

236 *Male and female anatomy*

237 The aedeagus consists of two structures: the phallus and the parameres ([26][28]; Figure 1).

238 The parameres are paired and lie either side of the phallus. They do not enter the female

239 tract during mating, but do appear to contact the external female genitalia and so may have
240 some stimulatory function during mating [26]. The phallus can be subdivided into three
241 main sections: the non-intromittent basal section, the intromittent middle region which is
242 covered in sharp spines, and the terminal endophallus (or internal sac) which is only everted
243 once the phallus is inside the female tract [26]. The basal section of the phallus is thickest,
244 and ends at a triangular structure referred to as the end plate or flap ([26][28]; Figure 1).
245 This section does not enter the female during mating. The intromittent portion of the
246 phallus has a thinner diameter and is covered in a ring of sharp spines. We were able to
247 count 230 distinct spines on the 3D volume of the aedeagus seen in Figure 1b. The spines
248 are highly sclerotized (note the bright red colour in Figure 1), however their attachment to
249 the outer surface of the phallus is flexible, allowing them to fold when needed. When fully
250 everted the spines can be seen to lie in a ring around almost the entire circumference of the
251 phallus, with the exception of a break along the dorsal surface. The spines appear longest on
252 either side, and shorter at the dorsal and ventral surfaces. Previous studies have classed
253 spines as either dorsal (along the dorsal surface either side of the break) or ventral (on the
254 ventral and lateral surfaces), a distinction which appears to have some functional relevance
255 [12]. Finally, the endophallus is made of soft tissue which conforms to the shape of the
256 lumen of the female tract when everted. The ejaculate, encased in a spermatophore, is
257 released from a slit at the tip of the endophallus [26].

258

259 At rest the aedeagus is stored inside the male abdomen, and the entire phallus is inverted
260 so that the spines line on the inside surface pointing towards the lumen. Prior to mating the
261 aedeagus is first drawn out of the male body cavity, and the phallus is partly everted before
262 insertion into the female genital opening [26]. Notably, at this stage a crown of spines is

263 unfolded at the tip of the aedeagus, and the aedeagus appears to be inserted into the
264 female genital opening in this position [26]. Once the aedeagus has been inserted into the
265 female genital opening, the phallus is further everted, revealing the rest of the aedeagal
266 spines. The process of unfolding of the phallus and spines inside the female tract can be
267 seen in Figure 1a (Video S1). Lastly, the endophallus is everted inside the female tract. This
268 entire process happens rapidly: in three of the four pairs frozen after 60 seconds of mating
269 the spines were fully unfolded and the endophallus was fully everted (Figure 1c; Videos S2,
270 S3). Ejaculate transfer was seen to be occurring in all pairs frozen at or after 300 seconds
271 (see below), and in a previous study has been shown to begin after 2-3 minutes [34].

272

273 The internal female copulatory system consists of two regions: a tubular copulatory tract
274 which receives the male intromittent organ, and a blind sac called the *bursa copulatrix* (or
275 bursa) which receives the male ejaculate [26][28]. The walls of the female copulatory tract
276 are very thick and folded prior to mating [28]. In the posterior region of the tract are
277 openings to both the common oviduct and the spermathecal duct [26]. The walls of the
278 bursa are much thinner than those of the copulatory tract, and prior to mating are highly
279 folded, so that the volume of the bursa is very small ([26]; Figure 2). The female bursal teeth
280 lie on the dorsal surface of the anterior region of the copulatory tract, at the entrance to the
281 bursa ([28]; Figure 2a, Video S3). The number of teeth is variable, ranging from two to seven
282 for the 19 females scanned here, with an average of 4.26 (s.d.= 1.1, $N= 19$; Figure 2c). Using
283 a much larger sample, Cayetano *et al.* [28] report an average of 3.09 teeth (s.d.= 1.06, $N=$
284 80). Also at the entrance to the bursa are two large lobes, which have been suggested to
285 function to limit the backflow of ejaculate out of the bursa [26], though this could not be
286 determined in the present study.

287

288 During mating the aedeagus forces the lumen of the copulatory tract to expand, and the
289 folds in the tract lining straighten out to allow this (Figure 2b; Video S3). The majority of
290 aedeagal spines appear to fit between the large folds in the tract lining (Figure 2b). In all of
291 the pairs scanned the aedeagus did not penetrate deep enough to reach past the bursal
292 teeth or lobes into the main bursal region (Figure 2a). The bursa expands even more
293 drastically as the large volume of ejaculate is transferred (Figure 3). The two bursal lobes are
294 also separated greatly by this expansion. The ejaculate appears to solidify into a single
295 glutinous mass once it has been deposited in the bursa [26][35], and despite the low X-Ray
296 attenuation of ejaculate material, micro-CT scans clearly show that a thin outer envelope is
297 visible around the ejaculate within a few minutes of being in the bursa (Figure 3). We refer
298 to this structure as the 'spermatophore envelope'.

299

300 *Copulatory wounding*

301 We observed penetrating spines in only five out of 19 pairs, and in three of them only a
302 single spine was seen to be embedded in the female tract (mean= 3.33, s.d.= 3.83, $N= 5$).
303 These spines also tended to be located near to the base of the aedeagus (near to the female
304 tract opening during mating). Holes in the female tract were much more common, with at
305 least one hole seen in all pairs scanned (mean= 14.2, s.d.= 9.8, $N= 19$). In all pairs the
306 number of tract holes was greater than the number of penetrating spines. This suggests that
307 when spines do pierce the female tract they do so for a short time before being removed.
308 This could be caused by movement of the aedeagus or contraction of the female tract.

309

310 By flash-freezing mating pairs at different stages of copulation we can determine when tract
311 wounding occurs during mating, and examine how the onset of female kicking relates to the
312 timing of copulatory wounding. Pairs were frozen at one of four time points: 1) after one
313 minute (no kicking), 2) after five minutes (no kicking), 3) after 30 seconds of kicking, and 4)
314 after two minutes of kicking. We counted the number of holes in the female tract for 19
315 mating pairs in total (4 pairs for treatment 1 and 5 pairs for treatments 2, 3 and 4). Note
316 that female kicking only occurs in the latter stages of mating, so that pairs from treatments
317 three and four also mated the longest: the average mating duration of the four treatments
318 was 60 (s.d.= 0), 301 (s.d.= 2.24), 332 (s.d.= 68.79) and 457 (s.d.= 54.84) seconds for
319 treatments 1, 2, 3 and 4 respectively. In only two pairs was more than one aedeagal spine
320 seen to be penetrating the female tract lining at the time of freezing, though both were
321 frozen at the later stages of mating (Figure 4a). The average number of holes in the female
322 copulatory tract lining differed across experimental treatments, though the difference was
323 not statistically significant ($F_{3,15} = 3.18$, $P = 0.054$; Figure 4b). Total hole volume was also not
324 related to experimental treatment ($F_{3,15} = 2.01$, $P = 0.16$). There are several insights that can
325 be gained from the data on the timing of copulatory wounding (Figure 4b). First, there
326 appears to be a small amount of wounding caused by the intromission process, or the
327 unfolding of the phallus during the first 60 seconds of mating. Second, significant wounding
328 is present prior to the onset of female kicking at around five minutes. Third, there appears
329 to be little difference in the amount of damage sustained by females frozen before or after
330 the onset of kicking.

331

332 Overall then, the wounding of the female tract lining appears to occur primarily before the
333 onset of kicking. Importantly, this means that kicking does not appear to begin at the onset

334 of copulatory wounding, as would be expected if kicking was primarily a female adaptation
335 to reduce harm during mating (though we cannot rule out the possibility that kicking begins
336 after females perceive that the degree of wounding has passed a certain threshold). These
337 results support recent claims that kicking is ineffective at reducing the amount of wounding
338 females receive during mating [15][36]. Conversely, we also find no support for the
339 hypothesis that kicking increases the rate of copulatory wounding females receive [15]. This
340 is perhaps surprising given that kicking is also associated with vigorous movements of the
341 female abdomen which could potentially exacerbate aedeagal spine penetration. This
342 suggests that more subtle movement of the aedeagus whilst inside the female (or even
343 contraction of the walls of the female tract) is the primary cause of copulatory wounding.
344 Based on this evidence, we conclude that female kicking has no significant (positive or
345 negative) influence on the degree of copulatory wounding females receive during mating, in
346 contrast to the conflicting results seen in previous studies [3][15]. Rather, we suggest that
347 kicking is triggered once the size of the ejaculate passes a certain threshold [14]. This is
348 supported by two further lines of evidence. First, females begin to kick sooner when mating
349 with larger males, which transfer ejaculate at a higher rate [36]. Second, males have been
350 shown to transfer smaller ejaculates with successive matings [37] [38], but the onset of
351 kicking is later for females mated to previously-mated males [17] [34].

352

353 We note however that our results on the timing of tract wounding should be interpreted
354 cautiously, for several reasons. First, the sample sizes used are relatively small, especially
355 given the high variation in the number of tract holes seen as mating duration increases.
356 Second, we did not assess the degree of tract wounding in females following non-
357 interrupted matings, so we cannot be sure that further wounding may occur as a result of

358 the aedeagus being pulled out of the female tract, especially from the downward-facing
359 spines at the base of the aedeagus. However, previous studies have counted the number of
360 holes in the female tract after allowing matings to end naturally, and report averages of
361 between 4 and 18 holes per female [11]. These values are consistent with those reported
362 here for interrupted matings, and so suggest there is little extra copulatory wounding
363 associated with the termination of mating. Third, our experimental design is unable to
364 separate the effect of kicking duration from overall copulation duration. Removing female's
365 ability to kick, for example by ablating their hind legs [3][16], would allow us to compare
366 long matings with and without kicking. This would allow us to test whether the rate of
367 wounding changes because of female kicking.

368

369 Across all females we detected holes in the copulatory tract corresponding to almost the
370 entire circumference of the aedeagus (Figure 5). The distribution of holes roughly
371 corresponds to the areas on the aedeagus with the greatest number and longest spines:
372 with more holes occurring on the sides than on the dorsal and ventral walls. We note
373 however that because the aedeagus is likely to move slightly during mating we cannot
374 definitively identify which spines are responsible for which holes. No holes or spines were
375 seen to penetrate through the entire wall of the tract. We thus suggest that male seminal
376 fluid products are unlikely to be able to pass directly into the female haemolymph following
377 traumatic mating [12]. However, tract holes do appear to reduce the distance through the
378 tract wall that such substances have to travel (by around a half in some cases), and this may
379 significantly increase the proportion of material leaving the female tract.

380

381 Though traumatic mating in *C. maculatus* appears to impose significant fitness costs to
382 females [3][39], the number of wound sites in the female tract is low compared to the
383 number of spines covering the aedeagus: assuming that all males possess 230 spines (see
384 estimate above), and that each hole is caused by a single unique spine, females receive
385 between 1% and 19% of potential penetrations. Indeed, at the time of freezing the vast
386 majority of male spines were not embedded in tract tissue, but instead fit between the large
387 folds in the female tract tissue (Figure 2b). This emphasises the fact that the flexibility of the
388 female tract lining is probably very important in preventing wounding in the first instance.
389 Flexibility could be increased for example by increasing the number of folds in the tissue, or
390 by increasing the elasticity of the tissue itself. Thus the morphology of the female tract has
391 likely evolved to both prevent spines from causing wounding in the first place (by increasing
392 the wall flexibility), and to subsequently reduce the cost of wounding after penetration
393 occurs (by increasing the wall thickness).

394

395 *Function of bursal teeth*

396 One suggested function of the female bursal teeth is to limit the intromission of the
397 aedeagus during mating [28]. We observed direct contact between the teeth and the male
398 endophallus in 12 out of 18 pairs for which the endophallus had been everted (Figure 3).
399 However, the endophallus is made of soft, flexible tissue and so is probably unlikely to be
400 damaged by such contact. The consistent position of the aedeagus across all mating pairs
401 also means that the more posterior region of the aedeagus (which could be susceptible to
402 damage) is unlikely to contact the teeth during normal mating. This lack of an antagonistic
403 role for the bursal teeth is also supported by the fact that there is no difference in the
404 number, length or allometry of the teeth in populations evolving under a male-biased or

405 female-biased sex ratio [28]. Further, it is not clear why males would benefit from deeper
406 penetration given that the entrance to the spermathecal duct is close to the female genital
407 opening [26], meaning that sperm deposited near the far wall of the bursa will have further
408 to travel in order to successfully reach the spermatheca. For these reasons we suggest that
409 the bursal teeth do not function to limit the penetration depth of the aedeagus. Instead, we
410 suggest that during mating the aedeagus is simply positioned at the entrance to the bursa in
411 order to allow effective deposition of the spermatophore into the bursa.

412

413 Notably, slice images from several mating pairs show evidence of direct contact between
414 the bursal teeth and the spermatophore as it is being deposited. Notably, out of the 14 pairs
415 frozen during spermatophore transfer, we observed direct evidence of the bursal teeth
416 piercing the outer spermatophore envelope in five pairs (Figure S2). We also observed
417 indirect evidence of contact between the teeth and spermatophore in the form of triangular
418 indentations in the spermatophore envelope in another five pairs (Figure S2). This strongly
419 supports the hypothesis that the bursal teeth function to pierce the spermatophore
420 envelope and thus aid the release of sperm and seminal fluid [14]. Further, the position of
421 the teeth allows this piercing to take place immediately as the spermatophore is being
422 deposited.

423

424 We have presented strong evidence that the female bursal teeth are able to pierce the
425 outer envelope of the spermatophore as it is being transferred during mating. This
426 interaction has not been observed previously, and we suggest that such an observation
427 would likely be very difficult using traditional microscopy techniques, given the gelatinous
428 nature of the spermatophore. However, it remains unclear whether this piercing is required

429 for successful sperm release, or just facilitates it, or whether there are other processes that
430 are also involved in breaking down the spermatophore envelope. It is also not clear how this
431 process is influenced by the number or size of the bursal teeth. To our knowledge only two
432 previous studies have considered the female bursal spines in relation to sexual conflict in *C.*
433 *maculatus* [28][40]. Cayetano *et al.* [28] did not find a significant relationship between the
434 strength of sexual selection and bursal tooth number, length or allometry, although there
435 was significant variation in tooth length across the experimental evolution lines. This
436 suggests that bursal tooth morphology is not under strong sexual selection. Cayetano &
437 Bonduriansky [40] found that average bursal tooth length (but not tooth number) was
438 significantly higher in females raised on beans that had previously contained a single larva,
439 compared to females raised on fresh beans. This is the opposite pattern to that predicted
440 for sexually-selected traits that exhibit condition-dependence. However, this pattern could
441 potentially be an adaptive female response to the level of sexual conflict, if females use the
442 presence of previously-infested beans as a proxy for high population density (which leads to
443 strong sexual conflict) [40]. Importantly, it remains to be tested whether tooth number or
444 size influences female or male fitness in any way.

445

446 *Conclusion*

447 The present study is the first to examine in detail the interactions between male and female
448 genitalia during mating in the seed beetle *Callosobruchus maculatus*. This has given us
449 several novel insights into the mating biology of this model organism. For example, we show
450 that X-Ray micro-CT can be used to detect tract tissue damage due to traumatic mating,
451 without the need to wait for the melanisations of wound sites. This technique can thus be
452 used in conjunction with flash-freezing to examine how tract damage accumulates over time

453 during a typical mating bout. Importantly, we show that significant female tract wounding is
454 present before the onset of female kicking. There is thus some degree of temporal
455 separation between the onset of wounding and the onset of kicking, which supports recent
456 suggestions that kicking is not an effective female counter-adaptation to reduce copulatory
457 wounding in *C. maculatus* [15]. We also provide the first evidence that the female bursal
458 teeth are able to pierce the envelope of the spermatophore during mating, suggesting that
459 they function to aid in sperm transfer during mating. We also rule out the hypothesis that
460 the bursal teeth function antagonistically to limit the intromission of the aedeagus.

461

462 More generally, we show here that contrast-enhanced X-Ray micro CT is an effective and
463 versatile technique for visualising genital interactions during mating (including copulatory
464 wounding), and one which we believe is at present underused. Even in well-studied species
465 such as *C. maculatus*, the functional roles of male and female genital traits remain
466 neglected. This is a problem that needs to be addressed, as without detailed anatomical
467 studies (and careful experimentation) we are unlikely to be able to determine the proximate
468 mechanisms of selection acting on male and female genital traits [41]. Additionally, X-Ray
469 micro CT produces a virtual representation of the sample, which can be used to take a range
470 of measurements in two and three dimensions, including shape and volume. Importantly,
471 this technique could be applied to other species in order to examine copulatory wounding
472 before the female immune response has had time to fully respond, both in time and space.

473

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488

489 **Author contributions**

490

491 LRD conceived of the study, designed the experiments, collected all data, performed all
492 analyses and drafted the manuscript. LWS conceived of the study, designed the experiments
493 and drafted the manuscript.

494

495 **Competing financial interests**

496

497 We have no competing interests.

498

499 **Data availability**

500

501 Supporting data has been archived at Dryad (DOI: <http://dx.doi.org/10.5061/dryad.33243>).

502

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Figure legends

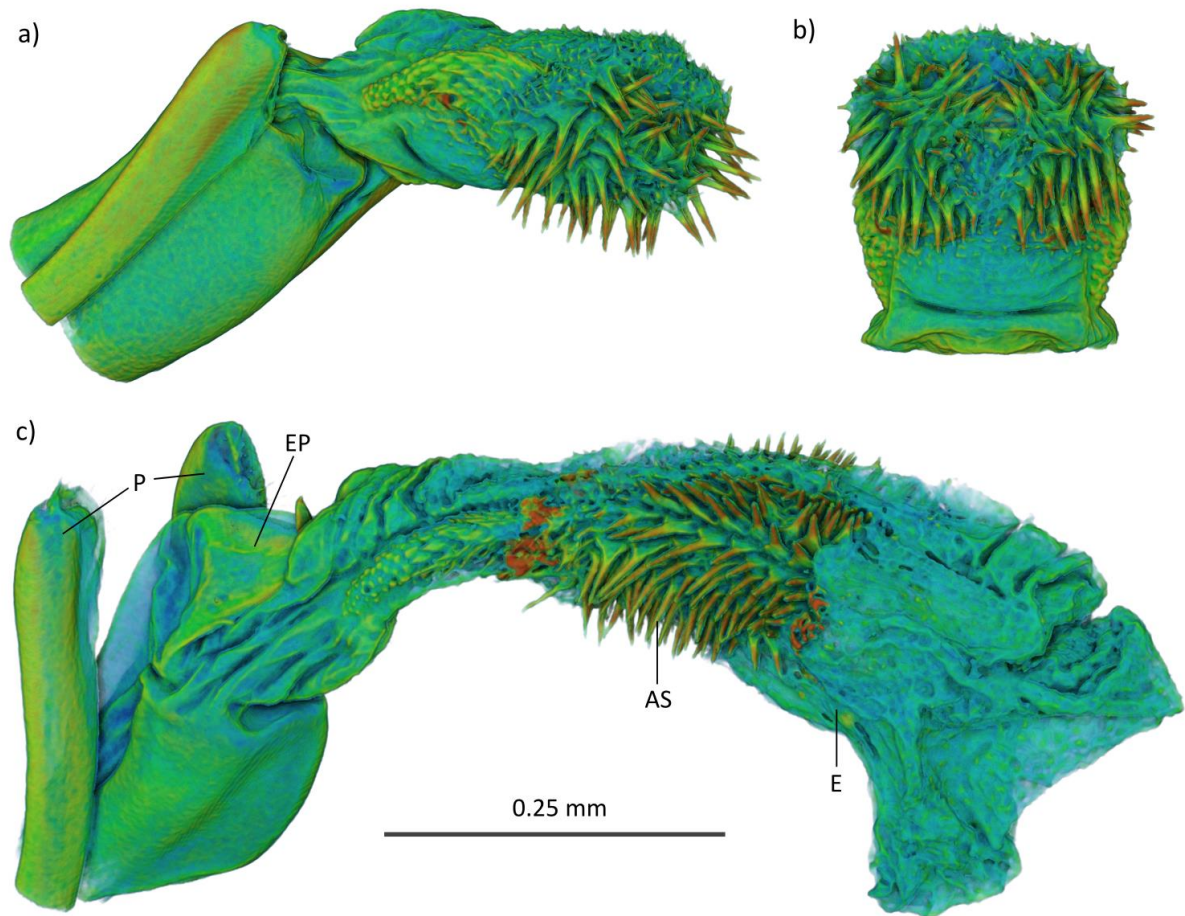


Figure 1. 3D volume rendering of the *C. maculatus* aedeagus during mating, obtained via X-Ray micro CT scanning. Panels a) and b) show two views of a partly everted aedeagus, and panel c) shows a fully everted aedeagus with the endophallus visible. Note that in both cases pairs were frozen in copula after 60 seconds of mating, so that the aedeagus is inside the female tract (not shown). The colour represents the relative X-Ray attenuation (brightness) of the tissue, with red representing highest density and blue representing lowest density. Abbreviations: AS: aedeagal spines, E: endophallus, EP: end plate, P: paramere. Note that the scale bar applies to panel c) only.

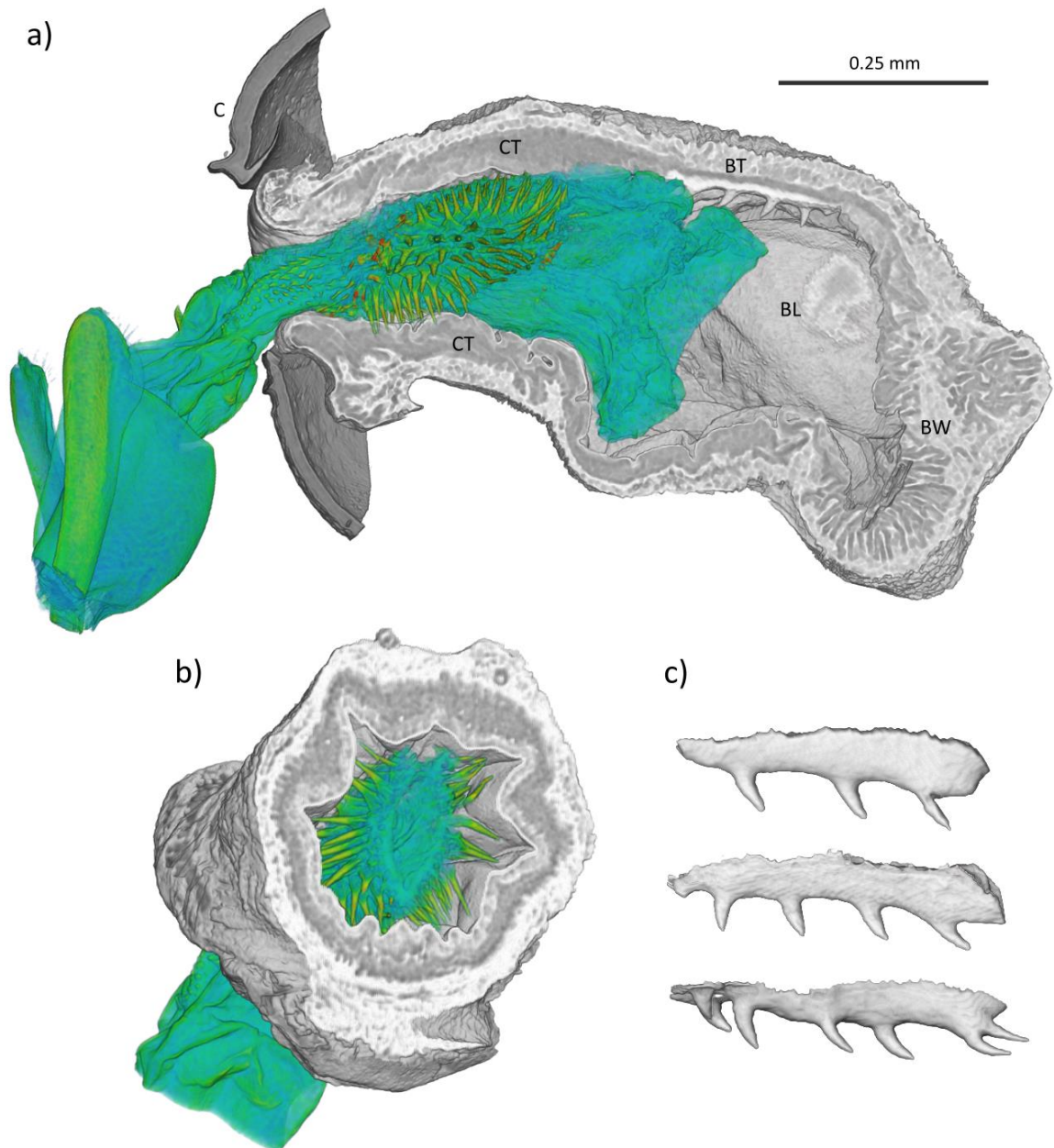


Figure 2. 3D volume rendering of the female reproductive anatomy of *C. maculatus*, obtained via X-Ray micro CT scanning. Panels a) and b) show the position of the aedeagus (colour) in relation to the female copulatory tract (grayscale) in a pair frozen after 60 seconds of mating, with virtual slices in the median (a) and transverse (b) planes respectively. Panel c) shows examples of bursal teeth from three females (not to scale). The colour represents the relative X-Ray attenuation (brightness) of the male tissue, with red representing highest density and blue representing lowest density. Abbreviations: BL: bursal lobe, BT: bursal teeth, BW: bursal wall, C: cuticle, CT: copulatory tract wall. Note that the scale bar applies to panel a) only.

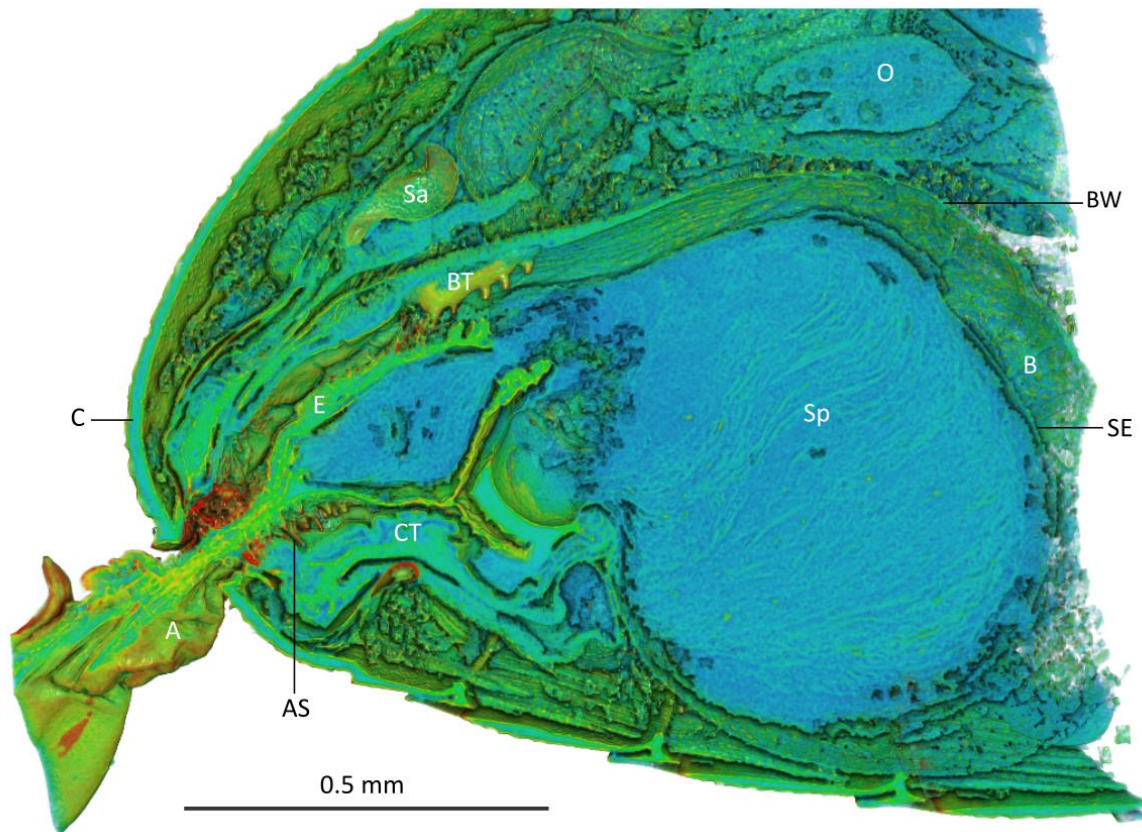


Figure 3. 3D volume rendering showing a median slice through the entire female abdomen during mating, obtained via X-Ray micro CT scanning. The pair was frozen after five minutes of mating, at which point spermatophore transfer is almost complete. The colour represents the relative X-Ray attenuation (brightness) of the tissue, with red representing highest density and blue representing lowest density. Abbreviations: A: aedeagus, AS: aedeagus spines, B: bursa lumen, BT: bursal teeth, BW: bursa wall, C: cuticle, CT: copulatory tract wall, E: endophallus, O: ovary, Sa: spermatheca, SE: spermatophore envelope, Sp: spermatophore.

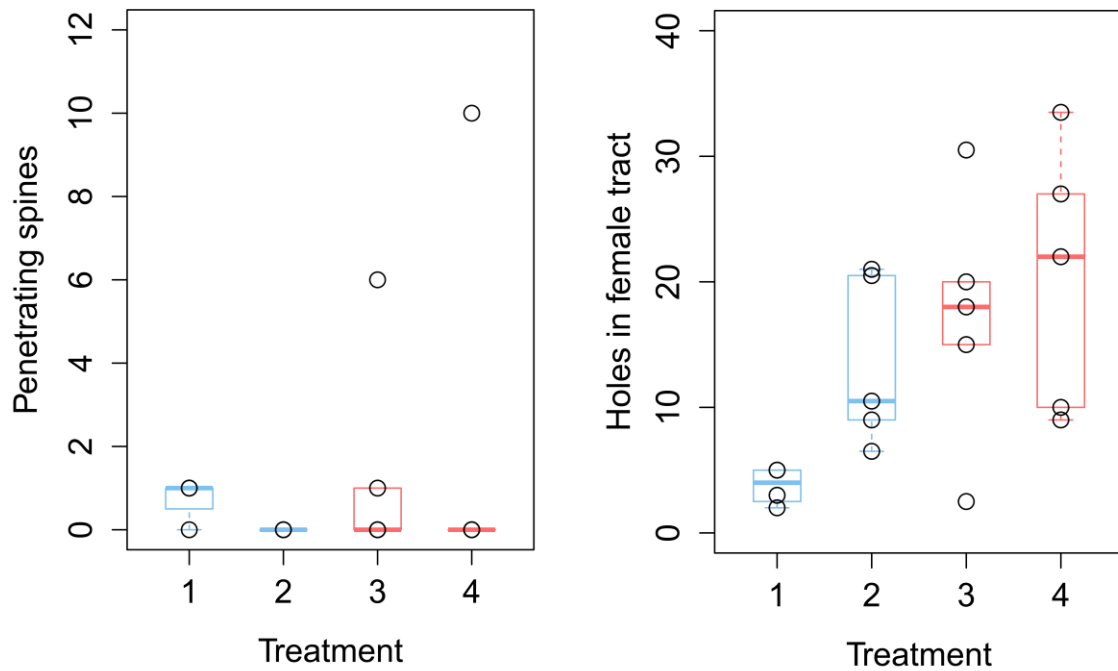


Figure 4. The timing of female tract wounding in *C. maculatus* during mating. Boxplots show differences in **a)** the number of penetrating spines and **b)** the number of holes in the female copulatory tract in relation to the four experimental freezing treatments. Pairs in treatments 1 & 2 were frozen prior to the onset of female kicking (blue boxes), and pairs in treatments 2 & 3 were frozen after the onset of female kicking (red boxes). The box height represents the interquartile range, and the whiskers represent 1.5 times the interquartile range above and below the box. See text for full treatment details and sample sizes.

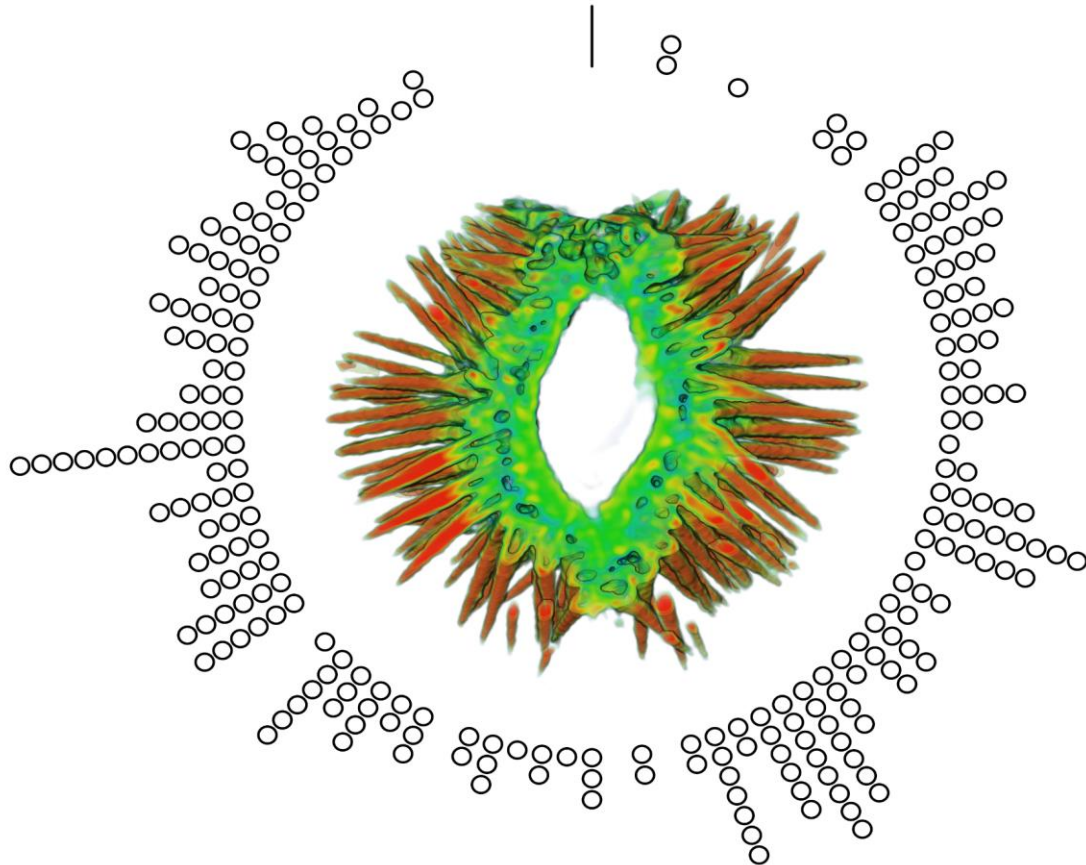


Figure 5. Radial plot showing the location of holes in the female reproductive tract lining in relation to the aedeagal spines along the anterior-posterior axis, across all mating pairs for which the aedeagal spines were fully everted ($N= 18$). Holes were counted in pairs frozen at various stages during mating (see text for details). In the centre of the plot is a representative example of an aedeagus in cross-section, showing the positioning of the spines around its edge.

Supplementary figure legends

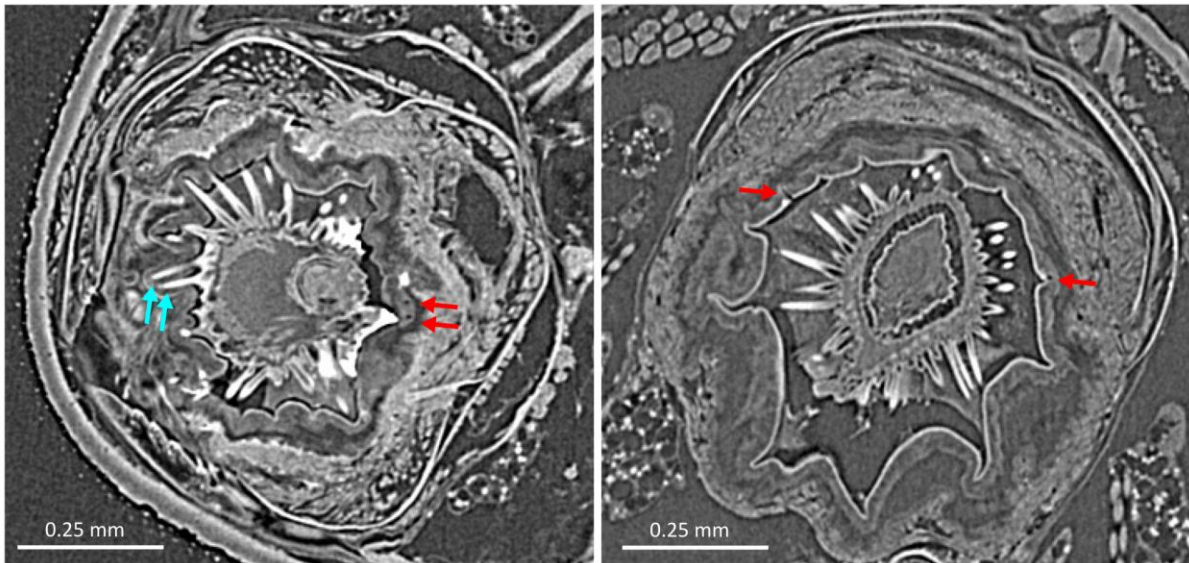


Figure S1. X-Ray Micro-CT slice images showing aedeagal spines embedded in the female copulatory tract at the time of freezing (blue arrows), and holes in the female tract lining purportedly caused by aedeagal spines (red arrows), in two pairs frozen after 505 minutes (left panel) and 445 minutes (right panel) of mating.

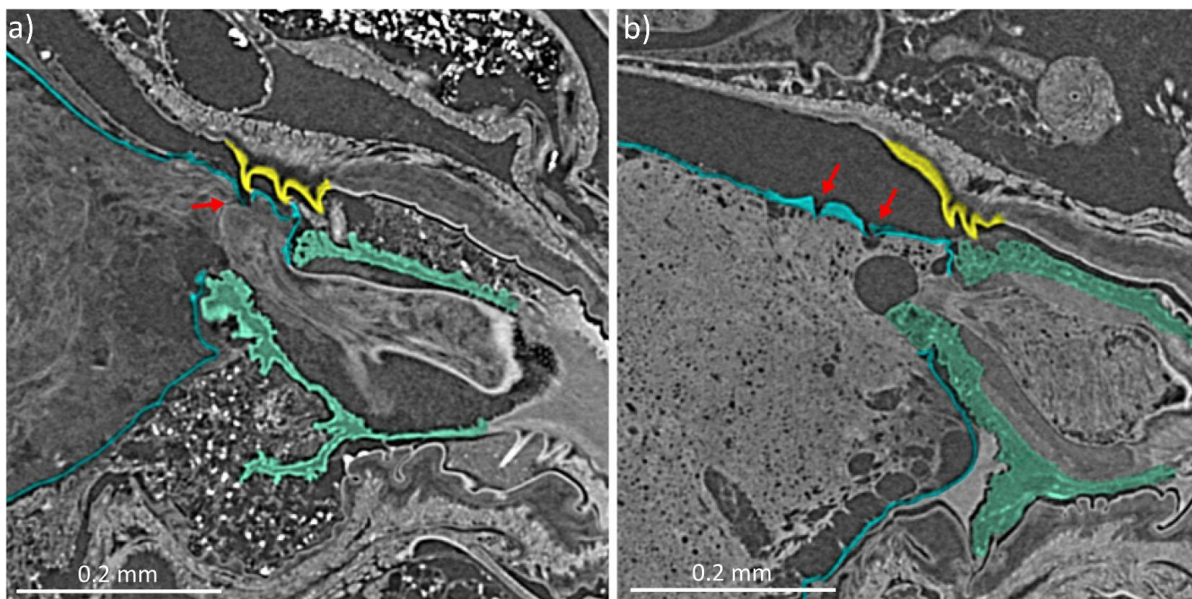


Figure S2. X-Ray Micro-CT slice images showing the interaction between the female bursal spines and the spermatophore during mating, showing a) piercing of the spermatophore envelope by the bursal teeth during insemination (red arrow), and b) indentations in the spermatophore envelope (red arrows) purported to be made during contact with the bursal teeth. In both images the female bursal teeth are highlighted in yellow, the aedeagus endophallus in green, and the spermatophore envelope in blue. Pairs were frozen after 260 minutes (a) and 505 minutes (b).