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X-Ray micro-CT scanning reveals temporal separation of male harm and female kicking during traumatic mating in seed beetles

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Running head: Copulatory wounding in seed beetles

Abstract

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

Keywords:

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

Introduction

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

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

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

Female *C. maculatus* may also have behavioural adaptations to reduce harm during mating. For example, females use their hind legs to kick males during the latter stages of mating [3][14][15]. It has been shown experimentally that female kicking reduces the duration of copulation [3][16], and that females able to kick sustain fewer tract wounds during mating [3]. It has therefore been traditionally assumed that kicking functions to dislodge the male sooner and thus reduce copulatory wounding. However, the results of two recent studies challenge this view. The first found that females able to kick their mates for longer showed reduced survival, and that both copulation and kicking duration increased when rival males were present, suggesting that these two traits are under male control to some extent [15]. The second found that copulation duration was repeatable when males and females mated repeatedly with the same mate (when no rivals were present), suggesting that copulation duration is the product of the interaction between males and female during mating [17]. These results suggest that female kicking on its own may be ineffective at reducing copulation duration. Further, the relationship between copulation duration and female fitness is complex: longer matings can lead to greater harm to females via copulatory wounding [3], but they also lead to the transfer of larger spermatophores which can directly and indirectly increase female fitness [18][19]. Accordingly, longer mating durations have been shown to both increase [14][18] and decrease [3][16] different measures of female fitness.

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

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

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

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

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 ± 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

X-Ray micro-CT scanning

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

Data analysis

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

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

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

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

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

Results and discussion

Male and female anatomy

The aedeagus consists of two structures: the phallus and the parameres ([26][28]; Figure 1). The parameres are paired and lie either side of the phallus. They do not enter the female

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

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

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

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

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

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

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

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

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

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

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

Function of bursal teeth

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

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

Conclusion

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

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

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

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Author contributions

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

Competing financial interests

We have no competing interests.

Data availability

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

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Figure legends

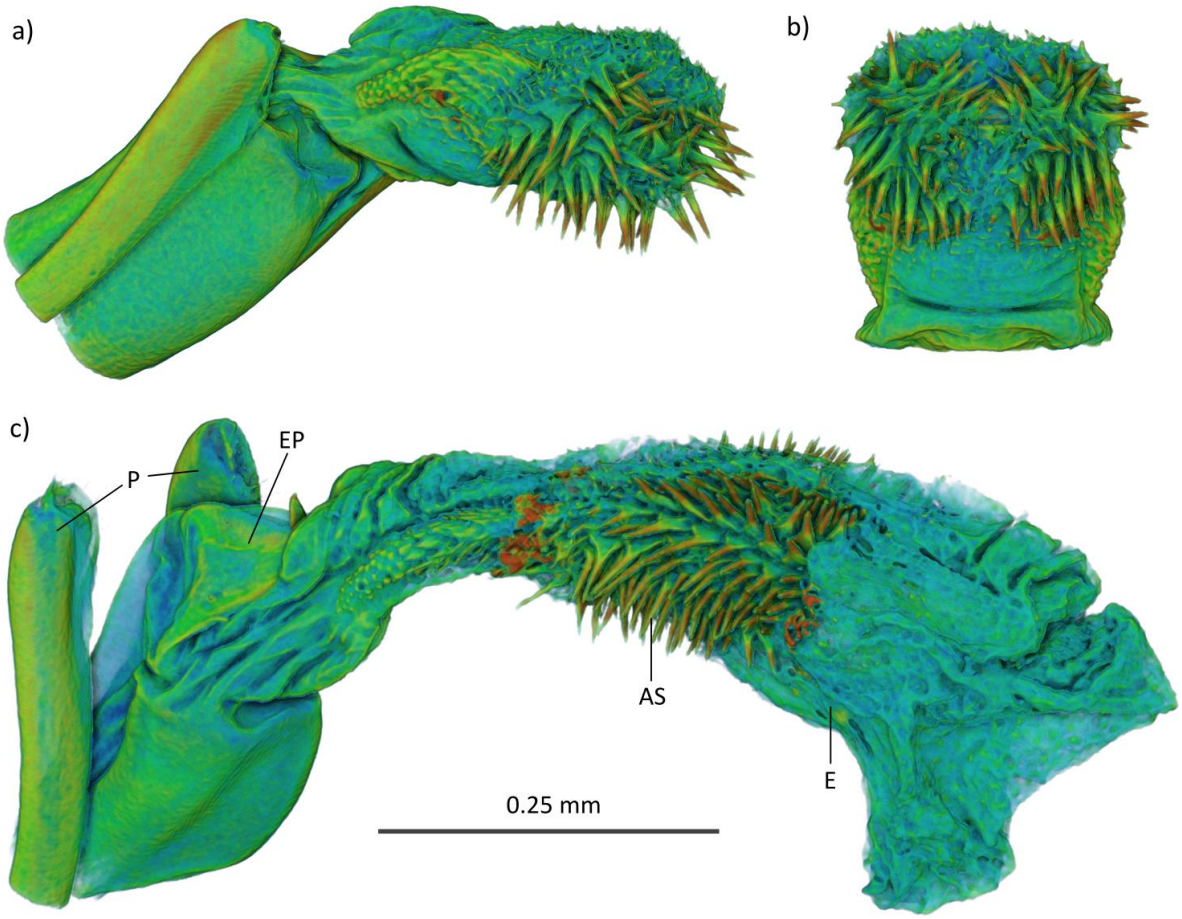

Figure 1. 3D volume rendering of the *C. maculatus* aedeagus during mating, obtained via X-Ray micro CT scanning. Panels a) and b) show two views of a partly everted aedeagus, and panel c) shows a fully everted aedeagus with the endophallus visible. Note that in both cases pairs were frozen in copula 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.

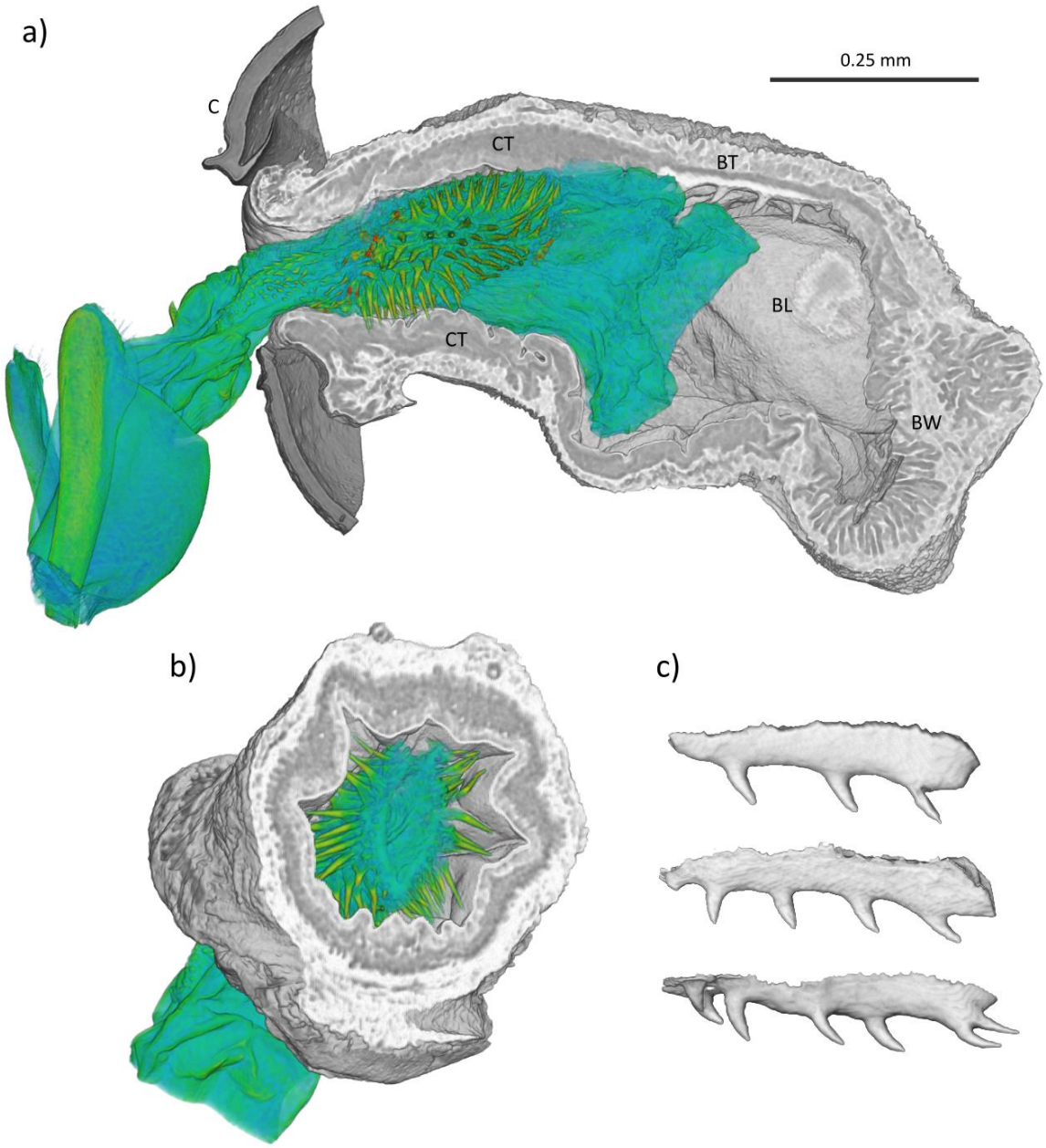

Figure 2. 3D volume rendering of the female reproductive anatomy of *C. maculatus*, obtained via X-Ray micro CT scanning. Panels a) and b) show the position of the aedeagus (colour) in relation to the female copulatory tract (grayscale) in a pair frozen after 60 seconds of mating, with virtual slices 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.

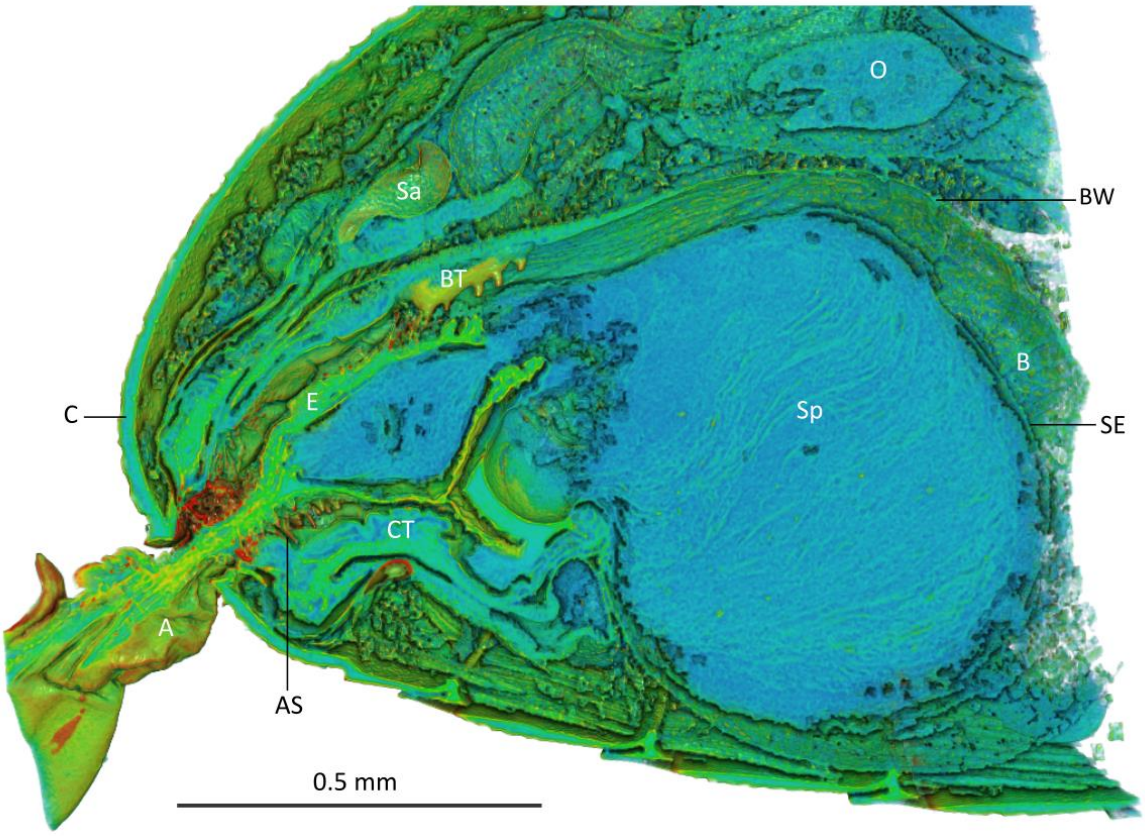

Figure 3. 3D volume rendering showing a median slice through the entire female abdomen during mating, obtained via X-Ray micro CT scanning. The pair was frozen after five minutes of mating, at which point spermatophore transfer is almost complete. The colour represents the relative X-Ray attenuation (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.

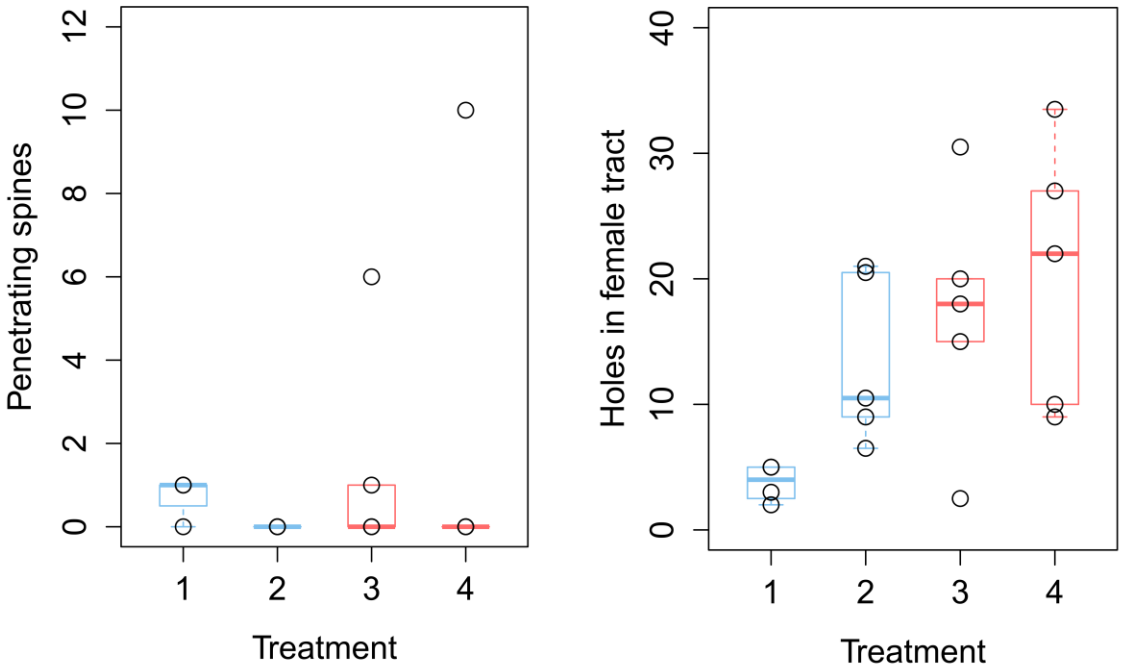

Figure 4. The timing of female tract wounding in *C. maculatus* during mating. Boxplots show differences in **a)** the number of penetrating spines and **b)** the number of holes in the female copulatory tract in relation to the four experimental freezing treatments. Pairs in treatments 1 & 2 were frozen 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.

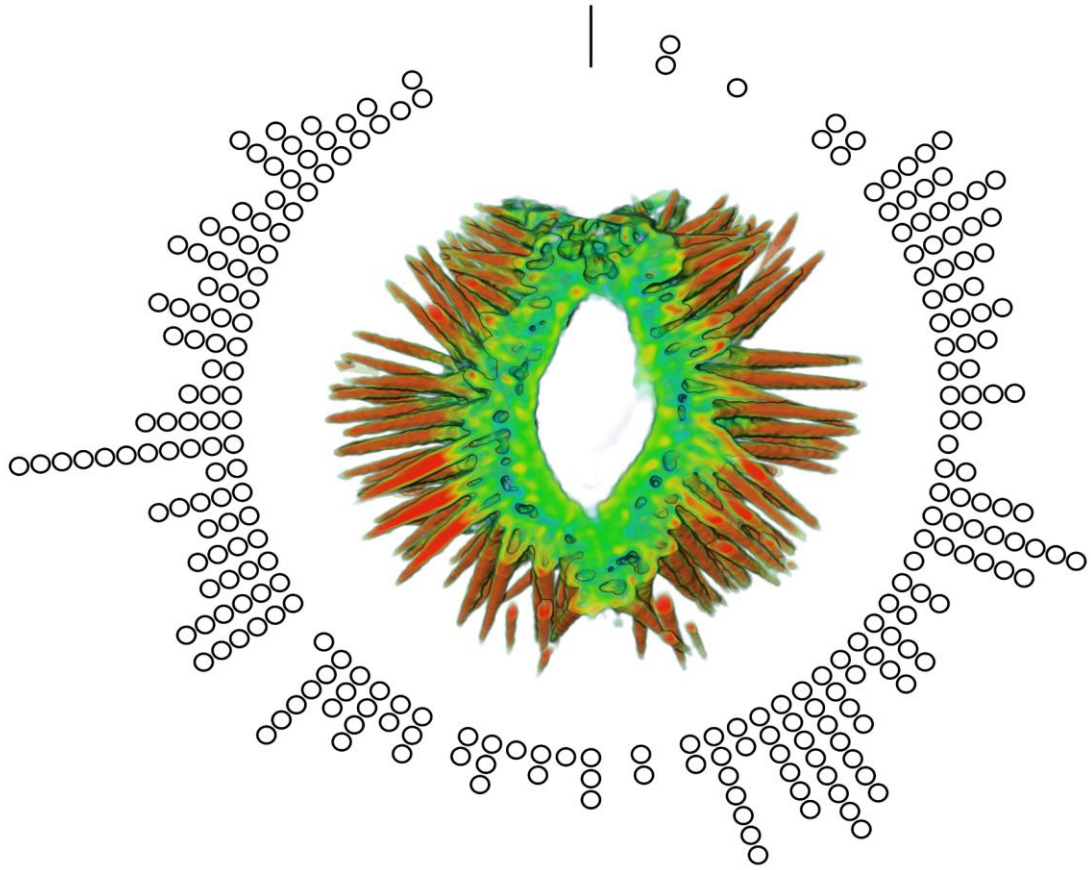

Figure 5. Radial plot showing the location of holes in the female reproductive tract lining in relation to the aedeagal spines along the anterior-posterior axis, across all mating pairs for which the aedeagal spines were fully everted (*N*= 18). Holes were counted in pairs frozen at various stages during 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

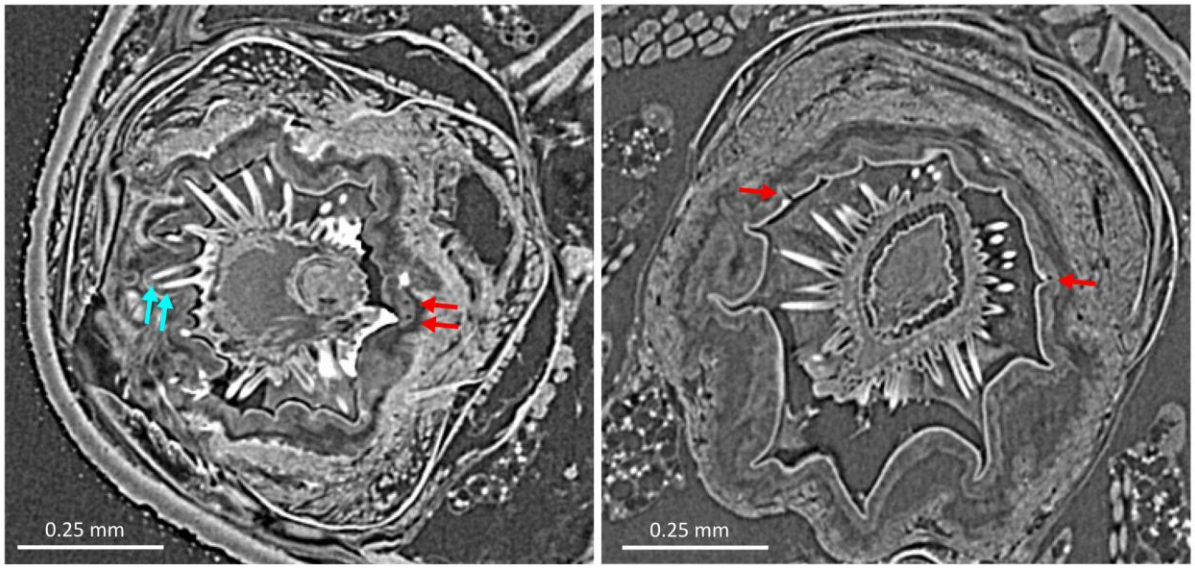

Figure S1. X-Ray Micro-CT slice images showing aedeagal spines embedded in the female copulatory tract at the time of freezing (blue arrows), and holes in the female tract lining purportedly caused by aedeagal spines (red arrows), in two pairs frozen after 505 minutes (left panel) and 445 minutes (right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

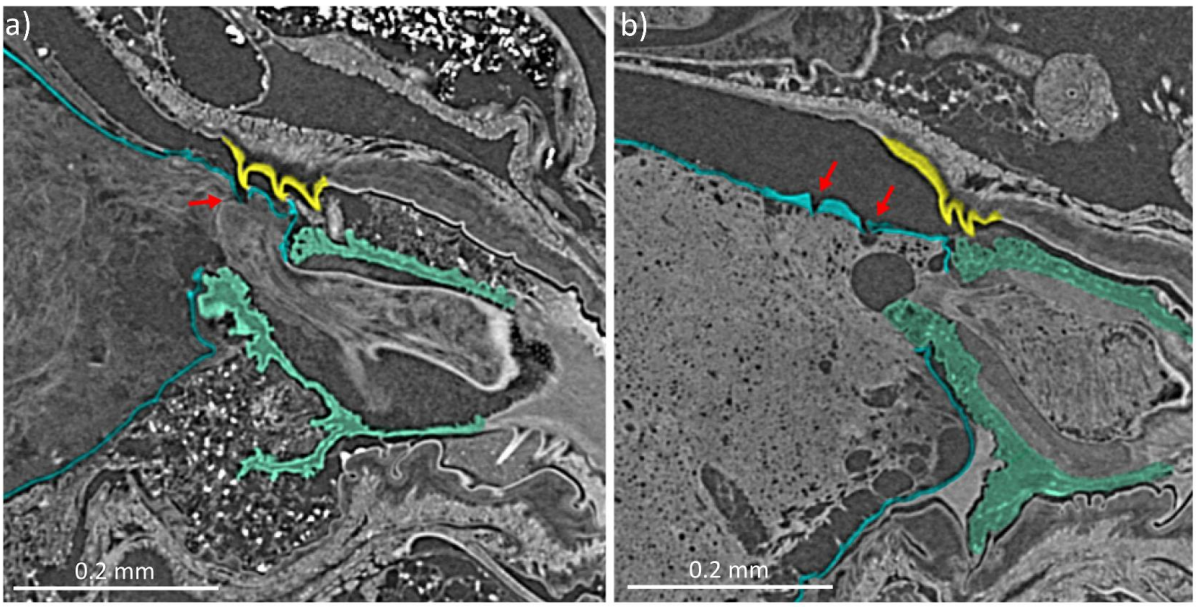

Figure S2. X-Ray Micro-CT slice images showing the interaction between the female bursal spines and the spermatophore during mating, showing a) piercing of the spermatophore envelope by the bursal teeth during insemination (red arrow), and b) indentations in the spermatophore envelope (red arrows) purported 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).