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Sexual conflict and correlated evolution between male persistence and female resistance traits in the seed beetle *Callosobruchus maculatus*

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5 Sexual conflict and correlated evolution between male persistence and female
6 resistance traits in the seed beetle *Callosobruchus maculatus*

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24

25 **Abstract**

26

27 Traumatic mating (or copulatory wounding) is an extreme form of sexual conflict whereby
28 male genitalia physically harm females during mating. In such species females are expected
29 to evolve counter-adaptations to reduce male-induced harm. Importantly, female counter-
30 adaptations may include both genital and non-genital traits. In this study, we examine
31 evolutionary associations between harmful male genital morphology and female
32 reproductive tract morphology and immune function across 13 populations of the seed
33 beetle *Callosobruchus maculatus*. We detected positive correlated evolution between the
34 injuriousness of male genitalia and putative female resistance adaptations across
35 populations. Moreover, we found evidence for a negative relationship between female
36 immunity and population productivity, which suggests that investment in female resistance
37 may be costly due to the resource trade-offs that are predicted between immunity and
38 reproduction. Finally, the degree of female tract scarring (harm to females) was greater in
39 those populations with both longer aedeagal spines and a thinner female tract lining. Our
40 results are thus consistent with a sexual arms race, which is only apparent when both male
41 and female traits are taken into account. Importantly, our study provides rare evidence for
42 sexually-antagonistic coevolution of male and female traits at the within-species level.

43

44 **Keywords**

45 *Callosobruchus*; genital coevolution; insect immunity; X-Ray micro-CT; sexual conflict;
46 traumatic mating

47

48

49 **Introduction**

50

51 Males and females may differ in their evolutionary interests, leading to sexual conflict over
52 the optimum expression of phenotypic or genotypic traits [1][2]. One of the most extreme
53 examples of sexual conflict is traumatic mating (also referred to as copulatory wounding),
54 whereby the male reproductive anatomy damages the female during mating [3]. This is
55 evidenced in many species by visible scarring of the female tract following mating (e.g.
56 [4][5][6][7]). The evolutionary advantage of such male harm has been the subject of
57 considerable debate. Males could benefit from harming females directly (the adaptive harm
58 hypothesis) if injury causes females to increase their short-term investment in reproduction
59 [8], or reduces their likelihood of remating [9]. However, empirical studies have revealed
60 little support for this theory (e.g. [10][11][12]), and it is now thought that trauma during
61 mating is a pleiotropic by-product of selection on genital traits that increase a male's mating
62 or fertilisation success [10][13][14].

63

64 Regardless of its evolutionary advantage to males, traumatic mating may negatively impact
65 female fitness (e.g. [4][15][5]). Thus, as with other forms of sexual conflict, the evolution of
66 harmful male traits is expected to drive the coevolution of defensive female traits which
67 minimise harm [2]. The result of this process is a positive correlation between the degree of
68 elaboration of harmful male traits and defensive female traits. Such a correlation has been
69 frequently demonstrated using interspecific comparisons (e.g. [16][17][18][19][20]), but has
70 only rarely been unveiled at the intraspecific level (e.g. [21][22][23] [24]). Detection of
71 correlated evolution at the species level is important for two reasons. First, different
72 processes may influence the outcome of sexually-antagonistic coevolution at the within-

73 species and between-species levels [22]. Second, micro-evolution occurs at the population
74 level, and so intraspecific studies are needed in order to link micro-evolutionary processes
75 to species-wide outcomes [22]. It is important to note that female resistance should
76 generally not be limited to single traits. Theory instead suggests that resistance in most
77 cases should be built by a suite of morphological, physiological and behavioural adaptations
78 acting together to reduce harm [2]. In these cases multivariate analyses, taking multiple
79 male and female traits into account, are most appropriate if we are to detect signs of
80 correlated evolution. This approach may be especially important in intraspecific studies, for
81 which the phenotypic differences in any single trait are typically smaller than in interspecific
82 comparisons.

83

84 The seed beetle *Callosobruchus maculatus* (Chrysomelidae; Bruchinae) is a model species
85 for the study of sexual conflict [25]. The male intromittent organ (aedeagus) is covered with
86 hundreds of sharp spines that penetrate and damage the walls of the female reproductive
87 tract during mating [4]. Males with longer aedeagal spines have increased competitive
88 fertilisation success [13], an effect which seems to be mediated via the passage of male
89 seminal fluid compounds into the female haemolymph, though it remains unclear whether
90 such passage occurs via wound sites [14]. There is some evidence that multiple mating
91 reduces female fitness in *C. maculatus* ([4][26][27], but see [25]), and one potential female
92 counter-adaptation to traumatic mating is a thickened reproductive tract lining [19]. This is
93 supported by the fact that there is a strong correlation between the degree of elaboration
94 of aedeagal spines and the thickness of the reproductive tract lining across seed beetle
95 species [19]. However, this relationship between male and female traits has not been shown
96 within any seed beetle species, nor has it been shown that variation in female tract

97 thickness influences the outcome of traumatic mating in *C. maculatus*. Females may need
98 physiological as well as morphological defences against copulatory wounding, if this
99 wounding for example increases the likelihood of microbial infection (e.g. [28][7]). In *C.*
100 *maculatus*, copulatory damage induces a rapid immune response by females to prevent
101 infection, resulting in the melanisation and plugging of damaged areas within 24 hours of
102 mating [4][26]. However, it is not clear how important female immunity is in mitigating male
103 harm in this species.

104

105 We examine covariation between three putative aspects of female counter-adaptation to
106 male-induced harm (one measure of female reproductive tract morphology and two
107 measures of female immune function) and male genital morphology and harmfulness across
108 13 laboratory populations of *C. maculatus*. These populations were collected in different
109 parts of the distributional range and have since been evolving independently in the
110 laboratory for more than a decade (which corresponds to >100 generations). Males vary
111 across populations in their average aedeagal spine length, and also in the amount of
112 copulatory damage their genitalia inflict on common standard reference females [13].
113 Previous work with these populations has also demonstrated covariation among
114 populations in aedeagal spine length and male competitive fertilization success [13].
115 Therefore, given that there is substantial variation in harmful male traits present across
116 these populations, we expect to see significant between-population variation in female
117 resistance traits as well.

118

119 We use micro-CT X-Ray scanning to measure the amount of tissue in the female
120 reproductive tract in three dimensions along the entire region contacted by the male

121 aedeagal spines. This approach allows us to control for any differences in the shape of the
122 tract which may be missed when using a small number of histological slices. If the lining of
123 the reproductive tract protects against traumatic mating, then we expect to see a positive
124 correlation between tract thickness and male persistence. We took two measures of female
125 immune function: phenoloxidase (PO) level and lytic activity. Phenoloxidase is an important
126 component of the insect immune system, performing a key role in wound repair and the
127 encapsulation and melanisation of foreign objects such as microbial cells [29]. The lytic
128 activity measures the efficacy of antibacterial peptides in the haemolymph. Both of these
129 immune traits are predicted to increase as the level of copulatory damage increases.

130

131 We use a multivariate statistical approach to test for a positive correlation between these
132 three female resistance traits (female tract volume, female PO level and female lytic
133 activity) and three male traits that collectively describe male persistence (see below). We
134 then use multivariate models to test whether the relative level of female resistance and
135 male persistence [30] in a population influences the degree of harm females receive during
136 mating. Showing such an effect would support the hypothesis that sexual conflict, rather
137 than some other process, has driven correlated evolution between males and females. Here,
138 we use the area of melanised scar tissue in the female reproductive tract lining, following
139 mating with males from their own population, as a proxy for female harm [4][26]. Finally, by
140 using previously-measured estimates of population-level growth rate (which is to a large
141 extent determined by female lifetime fecundity) for the 13 populations, we examine
142 whether investment in resistance traits significantly influences this measure of population
143 fitness.

144

145 **Methods**

146

147 *Populations*

148

149 Beetles from 13 established laboratory populations were used: Benin, Brazil/USA, California,
150 Mali, Nigeria/Lossa, Nigeria/OYO, Nigeria/Zaire, Oman, Uganda, Upper Volta, IITA, South
151 India, and Yemen. These populations were sourced from the wild and were brought into the
152 laboratory at different times. They are all laboratory-adapted, having been kept in
153 controlled conditions for at least 10 years, and have been used in several intraspecific
154 studies (e.g., [31][13][32][33][34][35]). All beetles used were reared on black-eyed beans
155 (*Vigna unguiculata*) and maintained under constant conditions at $30 \pm 0.5^\circ$ and $60 \pm 10\%$ RH
156 with a 12:12 h L:D cycle. We stress that all data presented here were gathered under
157 common garden conditions, such that significant difference between populations must
158 represent genetic differences. Further, previous studies have demonstrated a general lack of
159 phylogenetic signal in variation in reproductive phenotypes across these populations
160 [32][35]. Thus, we interpret phenotypic correlations across populations as representing
161 correlated evolution.

162

163 Here, we test for a multivariate association between three traits in females (reproductive
164 tract volume, phenoloxidase activity, lytic activity) and three traits in males (length of
165 ventral genital spines, length of dorsal genital spines, genital injuriousness). In addition, we
166 assess whether these traits relate to copulatory wounding and population fitness.

167

168 *Female reproductive tract volume*

169

170 One day old virgin females from each of the 13 populations were euthanized and then
171 weighed to the nearest 0.01 mg using an electronic balance (Sartorius Genius ME 235P-
172 OCE). The abdomen was then removed and stored in phosphate-buffered formalin in order
173 to fix tissues. Samples were stained in 1% Iodine in 100% ethanol (I2E: [36]) for 24 hours.
174 After staining samples were stored in 100% ethanol at room temperature, and scanned
175 between 1 and 12 weeks after staining. The order of scanning was randomized with respect
176 to the population of origin. Samples were scanned using a ZEISS Xradia Versa 520 X-Ray
177 microscope located at the University of Western Australia Centre for Microscopy,
178 Characterisation and Analysis. All samples were scanned using identical parameters (for
179 more detail see the supplementary methods), resulting in a voxel size of 2.35 μm . Scan data
180 was reconstructed using the XRADIA reconstructor package (XRADIA Inc). A total of 60
181 females were scanned across the 13 populations (4- 5 females per population).

182

183 The micro-CT data was analysed in two and three dimensions using Avizo 6 (FEI software).
184 All analyses were performed blind to the population origin of each sample. We manually
185 selected the area contacted by the spines of the aedeagus during mating ([19] Figure 1;
186 Figure S1). For full details see the supplementary methods. Once the entire region of
187 interest was selected, the number of voxels selected across all slices was then determined,
188 excluding the tract lumen, and converted into μm^3 to give a measure of the total volume of
189 tract tissue (Figure 1). We note that tract volume is thus a measure of overall investment in
190 reproductive tract tissue, taking into account both the thickness of the tract but also the
191 number of folds. A single observer performed the manual selection of the micro-CT data for
192 all females. To determine the repeatability of this manual selection, the tracts of ten

193 females were selected a second time using the same scan data but blind to the original
194 selections. Repeatability was determined using analysis of variance [37] and found to be
195 very high ($r= 0.993$).

196

197 *Female immune function*

198

199 At least 24 females from each of the populations ($N = 323$) were used for immune function
200 assays. Females were first weighed to the nearest 0.01 mg using an electronic balance
201 (Sartorius Genius ME 235P-OCE). Mean female weight per population was then used as our
202 measure of female size. Females were then gently crushed in a microtube in 20 μ l of
203 phosphate buffered solution (Amresco E404) (PBS). Samples were centrifuged at 0°C for 10
204 minutes at 17G, the supernatant was removed and then frozen at -80°C.

205

206 Phenoloxidase (hence, PO) level was measured using a method modified from [38]. For each
207 sample, 100 μ l PBS was added to 10 μ l of thawed haemolymph sample, and 100 μ l was then
208 pipetted into a 96-well microtitre plate. After adding 90 μ l 8 mM dopamine hydrochloride
209 (Sigma-Aldrich H8502), plates were loaded into a Tecan Infinite M200 plate reader (Tecan
210 Trading AG, Switzerland), where absorbance at 492 nm was measured every 5 minutes for
211 30 mins. This period was determined previously to be in the linear phase of the reaction. PO
212 activity (V_{max}) was measured as the maximum linear rate of substrate conversion.

213

214 To assay antibacterial activity, lytic zone assays were conducted. Agar plates were made
215 with 9 ml of 1% agar in which 5 mg ml⁻¹ of *Micrococcus luteus* (Sigma-Aldrich M3770) and
216 15 μ g ml⁻¹ streptomycin sulfate (Sigma-Aldrich S6501) was suspended [39]. Using a

217 sterilized Pasteur pipette, wells were punched in the agar. Into these wells, 2 μ l of
218 undiluted, thawed haemolymph sample were pipetted and incubated at 33°C for 24 h.
219 Zones of inhibition around each well were imaged under 10 \times magnification and measured
220 using ImageJ (version 1.48), with the area measured in pixels.

221

222 *Male traits*

223

224 Data on the average size of male aedeagal spines across the 13 populations were taken from
225 [13]. The spines are positioned on both the ventral and dorsal surface of the aedeagus.
226 Spine length was defined as the average length of the five longest spines for each male.
227 Average spine length was then calculated for each population ($N= 8-12$ males per
228 population). Hotzy & Arnqvist [13] also mated males from each population to females from
229 a common standard reference population, and the degree of tract scarring was then
230 measured using the same methods as in this study. This represents the degree of copulatory
231 wounding that standard “yardstick” females receive when they do not share recent co-
232 evolutionary history with their mates and, in this context, represents our third male trait
233 (hence; male injuriousness). For more details, we refer to [13].

234

235 *Reproductive tract scarring and population fitness*

236

237 To measure population differences in the amount of genital damage incurred by females
238 from different populations, 284 virgin females (20-24 from each population) were mated to
239 a virgin male each from within the same population. Mated females were then isolated for
240 24h to allow wound melanisation before being frozen in 70 % ethanol for later dissection.

241 We measured female body weight to the nearest 0.01 mg using an electronic balance
242 (Sartorius Genius ME 235P-OCE).
243
244 Preserved females were dissected in a drop of insect ringer (Grace's insect medium; Sigma-
245 Aldrich G81423). The female's bursa copulatrix was removed, cut along the midline and
246 spread onto a glass slide. The tract was then photographed at x400 and a digital image
247 recorded. Two measures of the damage to the tract were recorded: the total number of
248 differentiated wound sites (regardless of size), and the total combined area of melanisation
249 (sites of wound repair), which was measured using the outline tool of ImageJ (version 1.48).
250 Some degree of tract scarring was seen in all mated females.

251

252 Rankin and Arnqvist [31] quantified population fitness in these populations, as the per
253 generation growth rate in the absence of competition (total offspring produced by 10 males
254 and 10 females in a single generation). This is dictated primarily by female lifetime
255 fecundity, and we hence used this metric to assess population level costs of investment in
256 female immunity and resistance adaptations.

257

258 *Statistical analysis*

259

260 Statistical analysis were performed using R v3.2.2 [40], SYSTAT v.13.1 (Systat Software, San
261 Jose, CA) and Genstat v.18.1 [41]. We first used a GLM approach to test whether female
262 traits differed significantly across populations. In all models, female weight was included as
263 a covariate. For the tract scarring data, one pair was removed from the analysis because of a
264 coding error (N= 283 pairs in the final analysis).

265

266 To ask whether female resistance adaptations (three traits: female tract volume, female PO
267 level and female lytic activity) show correlated evolution with male persistence adaptations
268 (three traits: male dorsal spine length, male ventral spine length and male injuriousness),
269 we used two multivariate methods based on population averages for all traits. First, we
270 employed a canonical correlation analysis (CCA) to assess overall covariance between the
271 two sets of variables. Second, we used partial least-squares modelling (PLS) to achieve much
272 the same goal. Both of these methods assess covariance between a linear combination of
273 one set of variables with a linear combination of the other set of variables (i.e., a pair of
274 latent variables), thus capturing axes of covariation between the two sets of variables. The
275 relative contribution of different original variables to the latent variables can then be
276 gleaned by inspecting the loadings they have upon the latent variables. While CCA and PLS
277 analyses are related, they differ in how well they handle collinearity between original
278 variables within each set. In the population-level analyses, the covariance between traits
279 and size were removed by treating male size (elytra length) and female weight as partials.
280 Following regressions of each trait on size/weight (population means), residuals were
281 retained for analyses. We note that (i) this was deemed preferable to avoid
282 overparameterization of our inferential models but that (ii) analogous models instead using
283 raw trait values with size/weight included were qualitatively identical to the models
284 presented here.

285

286 **Results**

287

288 *Female traits*

289

290 The 13 populations differed significantly in average female tract volume ($F_{12,46} = 4.16$, $P <$
291 0.001), PO level ($F_{12,309} = 3.16$, $P < 0.001$), lytic activity ($F_{12,309} = 8.45$, $P < 0.001$), tract scar
292 area ($F_{12,268} = 1.88$, $P = 0.04$) and tract scar number ($F_{12,268} = 4.26$, $P < 0.001$). Across all
293 individuals, heavier females had a larger reproductive tract volume ($F_{1,46} = 15.05$, $P < 0.001$),
294 higher PO level ($F_{1,309} = 36.7$, $P < 0.001$) and higher lytic activity ($F_{1,309} = 7.14$, $P = 0.008$).
295 There was no effect of female weight on tract scar area ($F_{1,268} = 0.46$, $P = 0.5$) or scar
296 number ($F_{1,268} = 3.1$, $P = 0.08$). Females had an average of 17.84 scars (sd= 12.77) in the
297 tract wall.

298

299 *Correlated evolution between male and female traits*

300

301 The CCA revealed an overall covariation between the male and the female trait sets
302 (canonical $r = 0.93$; Rao's $F_{9,14.7} = 2.86$, $P = 0.035$) of which the first pair of latent variables
303 were significant ($\chi^2_9 = 18.27$, $P = 0.032$). A sizeable fraction of variance in female traits was
304 predicted by variance in male traits (Stewart-Love Canonical Redundancy Index = 0.57)
305 (Figure 2). Our PLS analysis also identified a single significant axis of covariation between
306 male and female traits (Osten's $F_{3,36} = 5.35$, $P = 0.004$), which explained 43.1% of the
307 variance in female traits and 42.9% of the variance in male traits. Inspections of the
308 standardized loadings of the two types of models (Figure 3) showed that the CCA and the
309 PLS were highly congruent, in terms of identifying very similar multivariate axes of
310 covariation. In males, the length of the dorsal spines and genital injuriousness contributed
311 to correlated evolution with females. In females, all three traits loaded positively upon the
312 female latent variable, although lytic activity did so most strongly (Figure 3). Overall, these

313 analyses support our predictions in terms of the pattern and direction of correlated
314 evolution between these putative male persistence and female resistance traits.
315
316 The amount of scarring represents an outcome of a male-female interaction and so should
317 not be affected by male persistence or female resistance in isolation, if male-female
318 coevolution is balanced [30]. We tested whether the amount of scarring in females that
319 resulted from within-population matings covaried with either male persistence or female
320 resistance by correlating our population-specific measures of scarring (number and area)
321 with population scores along the latent variables of the CCA and the PLS. As predicted,
322 scarring showed no significant correlation with either of our male or female traits in
323 isolation ($|r| < 0.45$, $P > 0.125$, in all cases). Theory predicts, however, that the outcome
324 could be predicted in a multivariate analysis where male and female traits are used
325 simultaneously [30]. A model using all 6 male and female original traits to predict scar area
326 was not significant overall ($F_{6,6} = 0.54$, $P = 0.761$) but a model predicting scar number was ($F_{6,6} = 5.70$, $P = 0.026$). Because the model of scar number was potentially overparameterized
327 and suffered from problems with multicollinearity, we also assessed the model using (1) a
328 resampling test involving bootstrapping (10^3 replicates) the regression coefficients and (2) a
329 ridge regression using both the Hoerl-Kennard-Baldwin (HKB) estimator and the Lawless &
330 Wang (LW) estimator of lambda. These assessments (Table 1) showed that the initial model
331 was robust against the above potential problems and that two variables showed
332 independent effects on the number of female scars: injury to females was higher in
333 populations where males had size-corrected ventral genital spines that were long relative to
334 the reproductive tract volume of females (Figure 4).
335
336

337 *Female resistance and population fitness*

338

339 Multiple regression suggested that female investment in resistance (i.e., the score along the
340 female latent variable) and female size collectively predicted population fitness when using
341 latent variable scores from the PLS ($F_{2,10} = 4.19, P = 0.048$), but not from the CCA ($F_{2,10} =$
342 $3.44, P = 0.073$). A closer inspection of this pattern showed that the covariation was
343 primarily due to a negative correlation between population fitness and female PO level ($r =$
344 $-0.59, P = 0.032$), rather than reproductive tract volume ($r = -0.38, P = 0.199$) or lytic
345 activity ($r = -0.04, P = 0.901$). These analyses thus offer support for the hypothesis that
346 female investment in at least some aspects of resistance are costly in terms of reduced
347 population fitness [19][42].

348

349 **Discussion**

350

351 In this study we examined across-population covariation in male persistence traits and
352 female resistance traits using 13 populations of the seed beetle *C. maculatus*. We found
353 significant across-population differences in all of the female traits measured, indicating that
354 these traits have diverged significantly in isolation. Multivariate analyses revealed significant
355 positive correlated evolution between male persistence and female resistance adaptations
356 across populations. Our study thus provides a rare example of correlated evolution of male
357 persistence and female resistance traits at the within-species level [22], and illustrates the
358 importance of considering multiple traits given that male and female adaptations to sexual
359 conflict are unlikely to be limited to single traits.

360

361 In order to show that the correlated evolution between male and female traits observed
362 here is caused by sexual conflict, we need to demonstrate that an increase in male
363 persistence is associated with a reduction in female fitness [2][43]. Yet, when such a ‘sexual
364 arms race’ is present we should not expect to find a direct relationship between the level of
365 male persistence and female fitness, as any reduction in female fitness should quickly lead
366 to an increase in female resistance traits to reduce harm [30]. Indeed, when traits were
367 tested in isolation, we found no significant effect of male persistence or female resistance
368 on the degree of tract scarring across populations. This is consistent with a scenario where,
369 within each population, males and females are at an evolutionary equilibrium with respect
370 to the fitness impact of traumatic mating. However, the hallmarks of such an arms race may
371 be detected by considering the levels of both male and female adaptations simultaneously
372 [30][19]. Our multivariate analyses revealed that male ventral spine length and female tract
373 volume significantly influenced the number of scars in the female tract (Table 1), although
374 there was no significant effects on tract scar area. Female tract scarring was highest in those
375 populations with relatively long ventral spines and relatively small average female tract
376 volume (Figure 4.). Further, for most populations the level of investment in ventral spine
377 length is roughly matched by the level of investment in reproductive tract volume. This
378 provides support for the ‘arms-race’ hypothesis for the evolution of male genital spines and
379 female tract thickness: differences in the absolute level of any male or female trait do not
380 influence the fitness outcomes of mating, whereas differences in the relative level do
381 [30][19][22]. As well as providing evidence for a sexual arms race, this statistical approach
382 has also allowed us to confirm intraspecifically for the first time that both male aedeagal
383 spine length and female tract thickness do indeed influence the outcome of traumatic
384 mating in *C. maculatus*.

385

386 It is important to note that we have used a three-dimensional measure of female tract
387 tissue investment in this study, rather than a simple measure of the thickness of the tract in
388 cross-section. Given that the female tract is a three-dimensional structure, we suggest this
389 three-dimensional measurement is the most appropriate when considering the fitness
390 effects of traumatic mating, as it most fully captures differences in total female investment
391 in tract tissue. This method also controls for any confounding effect of tract size or shape
392 across females, which could be overlooked when only taking tract thickness estimates from
393 one or a few transverse slices through the tract (e.g. [19][44]). However, the use of tract
394 volume makes determining the precise proximate mechanisms leading to changes in female
395 fitness more difficult. For example, it has been suggested that a thicker tract lining reduces
396 the cost of mating to females by reducing the amount of male-seminal products that are
397 able to pass into the female body cavity [14]. However, tract volume could be increased in
398 two ways: by increasing the tract thickness, or by increasing the number of folds in the tract
399 lining (as seen in Figure 1a). The number of folds in the tract lining could also feasibly reduce
400 tract scarring, and thus the fitness costs of mating to females, by giving the tract lining
401 greater flexibility and so making the penetration of spines more difficult. Therefore, we
402 cannot distinguish between the effect of physical distance between the tract lumen and the
403 body cavity, or some other effect such as the number of folds, based on the relationship
404 between tract volume and female fitness alone. Instead, functional studies of the
405 interaction between the male and female genitalia are needed. This is an area in which
406 micro-CT scanning may prove very useful, and indeed this approach has been used
407 effectively to examine the interactions between male and female genitalia during copulation
408 in other arthropod species (e.g. [45][46][47]).

409

410 We found strong evidence for correlated evolution between male genital morphology and
411 both measures of female immune function, with female lytic activity showing the strongest
412 covariation with male persistence traits. This supports the hypothesis that the female
413 immune response has evolved to reduce the cost of traumatic mating in *C. maculatus*, with
414 microbial infection being a potential target of female resistance. However, neither measure
415 of female immunity was directly related to the degree of tract scarring seen following
416 mating. This is somewhat surprising, given that both lytic activity and phenoloxidase level
417 are predicted to play a role in reducing the costs of female tissue damage. However, the
418 female immune system has to respond to costs of mating other than those arising from
419 copulatory tract damage. For example, females may suffer mating costs via male seminal
420 fluid proteins that are known to vary across populations [48][49][35]. In addition,
421 investment in immunity by females is affected by a suite of other life history trade-offs in
422 seed beetles (e.g. [50]). Factors such as these are likely to blur the relationship between
423 scarring and immunity.

424

425 We also found evidence for a trade-off between one aspect of female immune investment
426 (PO level) and population fitness: populations with high female PO levels tended to have
427 lower population fitness. This is likely due to the fundamental resource trade-offs that are
428 predicted between immunity and reproduction [51][52], given that population fitness
429 primarily reflects differences in female egg production [31]. This trade-off represents an
430 additional, and under-appreciated, potential cost of traumatic mating to females that has
431 been seen in other studies of *C. maculatus* (see also [53][42]). Interestingly, a recent study
432 assessing lytic activity in *C. maculatus* populations subject to an experimentally biased sex-

433 ratio for 11 generations also found evidence for such a trade-off: females from male-biased
434 populations had lower lytic activity than females from female-biased populations [39].
435 Females in male-biased lines are predicted to experience an increased mating rate (and
436 therefore greater lifetime mating trauma), and so are expected to increase investment in
437 immunity. However, this is the opposite of the pattern observed by van Lieshout *et al.* [39].
438 Their result could be explained if there is a strong trade-off between investment in
439 reproduction versus immunity, such that females subjected to greater mating costs are
440 adapted to invest in early reproduction at the expense of immune function [39][50].

441

442 One outstanding question concerns the extent to which the differences in male and female
443 traits observed among the current laboratory populations reflect differences between the
444 ancestral populations from which they were collected, relative to subsequent divergence
445 among populations since they were introduced into the laboratory. Unfortunately,
446 determining this is not possible without a knowledge of the phenotypes of the ancestral
447 populations at the time when founder individuals were collected. We suggest that
448 laboratory divergence has been less important than the original population differences,
449 given that (i) all populations have experienced a single common garden environment, and
450 (ii) reproductive traits are not correlated with the time since collection across these
451 populations [31][35]. Indeed, if these populations are adapting to the same common
452 environment the differences we observe now are likely to be reduced compared with
453 ancestral populations. Regardless, the fact remains that there has been significant
454 correlated evolution between males and females across these populations, though the
455 timescale over which such differences have evolved is unclear.

456

457 In summary, by combining multiple morphological and physiological measurements we have
458 detected a clear signal of correlated evolution between male persistence traits and female
459 resistance traits involved in sexual conflict in the seed beetle *C. maculatus*. We have also
460 shown that the relative level of male and female “armament” influences the degree of harm
461 females receive during mating, thus providing support for the hypothesis that this
462 correlated evolution has been driven by sexually antagonistic coevolution. We have thus
463 shown that the process that has resulted in the covariation between male and female
464 phenotypes across seed beetle species is also ongoing within at least one of these species.
465 Finally, we present evidence for a trade-off between investment in female immune function
466 and reproductive function at the population level, thus providing evidence of an additional
467 cost to females of traumatic mating.

468

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470

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485

486 **Author contributions**

487

488 LRD performed the female tract volume measurements, carried out statistical analysis and
489 drafted the manuscript. EVL conceived the study, collected the beetles and performed the
490 immunity measurements. KBM conceived the study and helped draft the manuscript. JAM
491 performed the female tract volume measurements. GAS set up the original populations,
492 performed fitness assays, secured measures of male spines, performed statistical analysis
493 and helped draft the manuscript. LWS conceived the study, coordinated the study and
494 helped draft the manuscript. All authors gave final approval for publication.

495

496 **Competing interests**

497

498 We have no competing interests.

499

500 **Supporting data**

501

502 Supporting data has been archived at Dryad (<http://dx.doi.org/10.5061/dryad.1b15j>).

503

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634

635

636 **Figure legends**

637

638 Figure 1. Female reproductive tract morphology in *Callosobruchus maculatus*. Panel a)
639 shows a representative CT slice image of a female tract outlined in red, showing the thick
640 walls and dark lumen. Panel b) shows a three-dimensional volume rendering of a female
641 tract viewed laterally, created by combining multiple two-dimensional slices (note that a 3d

642 slice has been used to virtually cut the tract in half). In both cases brightness represents the
643 degree of tissue staining.

644

645 Figure 2. Ordination of the 13 populations along the first pair of latent variables describing
646 covariation between male genital injuriousness and female resistance traits. Closed symbols
647 represent scores from a canonical correlation analysis and open symbols those from a
648 partial least-squares model. See text for statistical details.

649

650 Figure 3. Loadings for the male set (left) and female set (right) of traits upon the two sex-
651 specific latent variables best describing correlated evolution between the sexes. Open
652 circles show loadings from the PLS model. Closed circles represent the CCA. Shown are also
653 bootstrapped 95% CI's for the CCA loadings, based on 10^3 bootstraps corrected for axis
654 reversals.

655

656 Figure 4. Heat map of the amount of genital damage incurred by females during mating
657 (number of scars in reproductive tract) as a result of variation in size-corrected male genital
658 spine length and volume of the female reproductive tract, across the 13 populations
659 (circles). Circle size is also proportional to the number of scars.

Table 1. The results of a multiple regression based on mean trait values across 13 populations, using three male traits (M) and three female traits (F) to predict the number of scars in the female reproductive tract that results from mating. Body size was partialled out from male and female traits prior to analysis. Significant effects are highlighted in bold. See text for discussion.

Variable	β	t	P	Bootstrap 95% CI*		Ridge β (HKB)	Ridge β (LW)
				Lower	Upper		
M: dorsal spine length	0.88	1.62	0.157	-3.46	2.52	0.89	0.77
M: ventral spine length	0.97	2.92	0.027	0.23	3.87	0.72	0.50
M: male injuriousness	-2.45E-03	-2.09	0.082	-0.01	0.02	-1.60E-03	-1.04E-03
F: reproductive tract volume	-0.59	-4.88	0.003	-1.74	-0.23	-0.51	-0.42
F: PO level	479.21	1.64	0.151	-2.05	2869.00	338.39	256.77
F: lytic activity	3.28E-05	1.12	0.306	-5.40E-05	2.61E-04	1.38E-05	3.61E-06

* Bias corrected

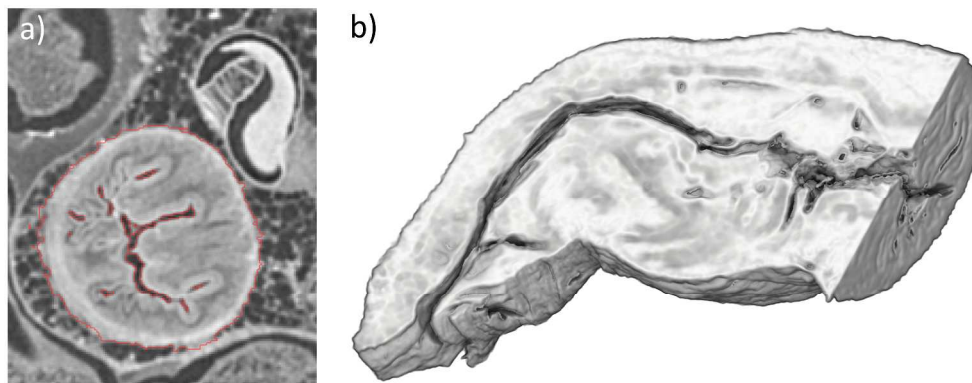
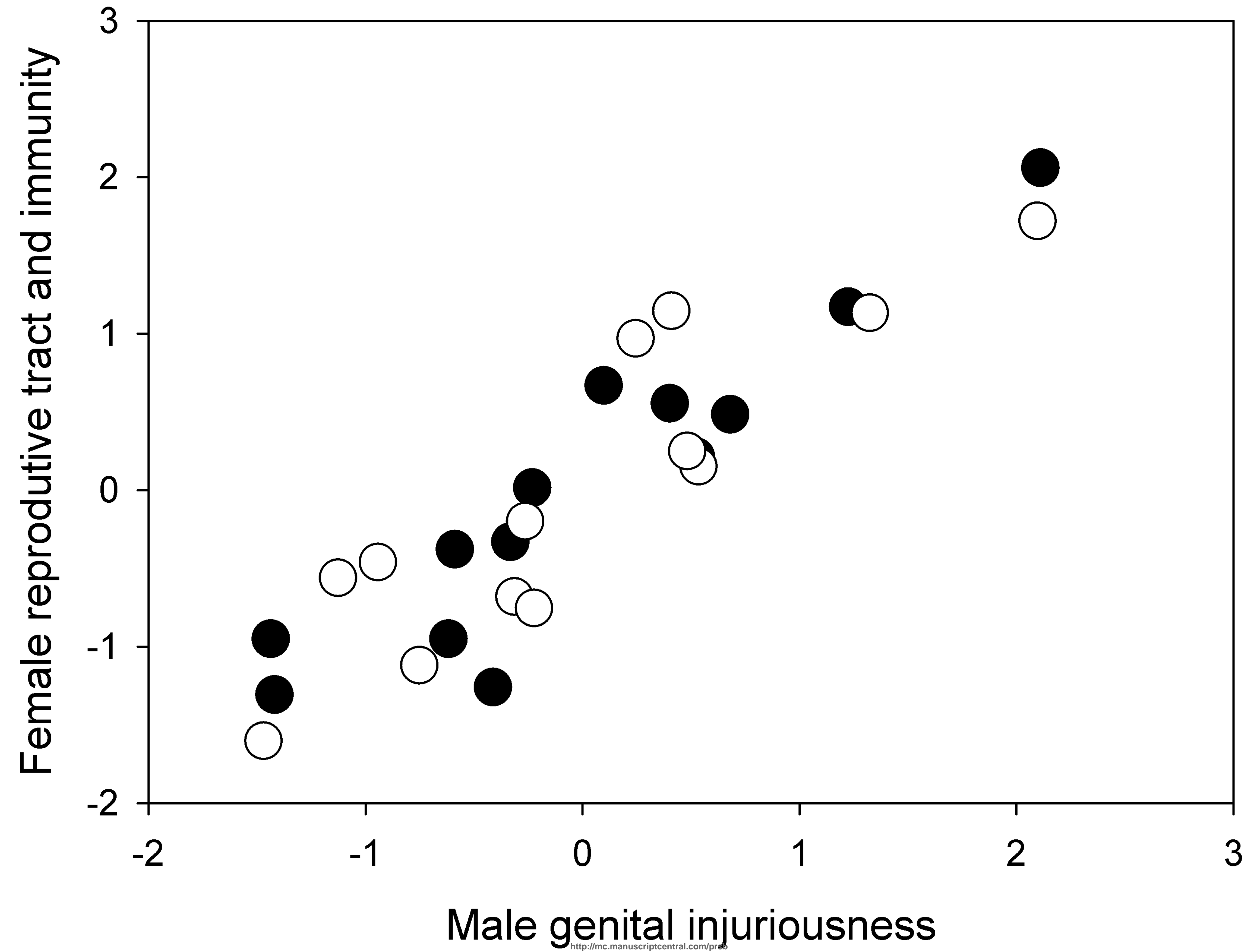
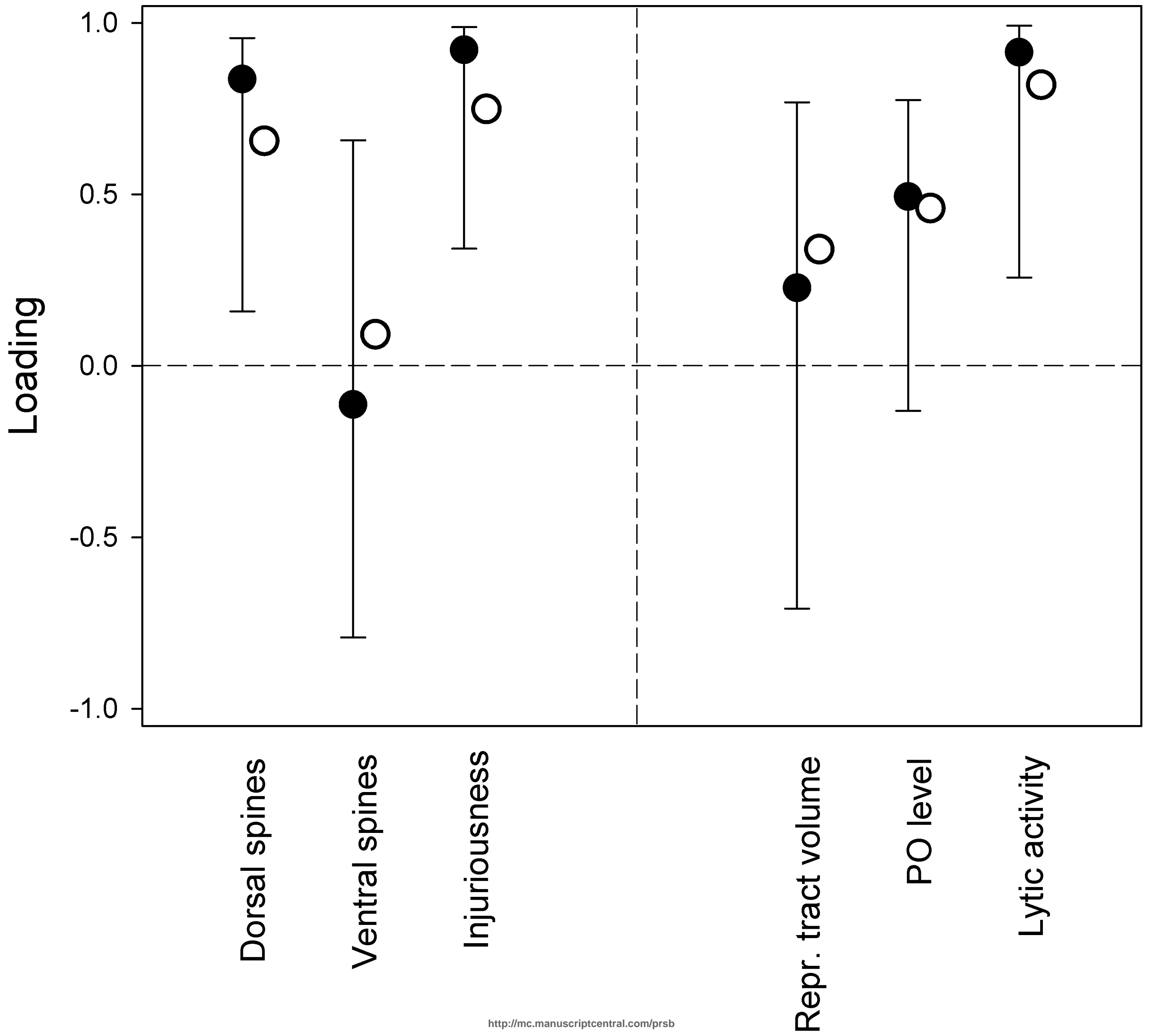


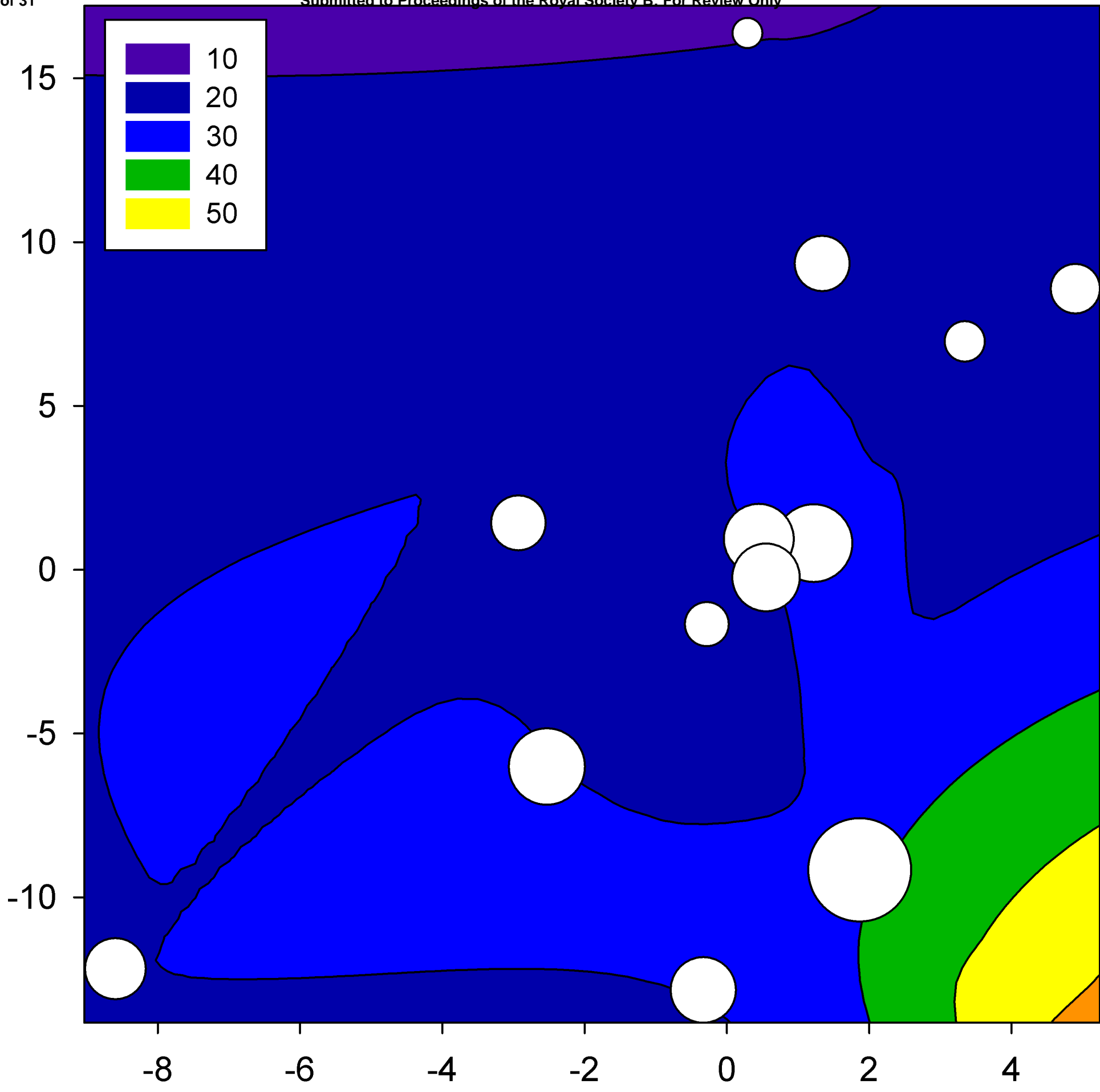
Figure 1. Female reproductive tract morphology in *Callosobruchus maculatus*. Panel a) shows a representative CT slice image of a female tract outlined in red, showing the thick walls and dark lumen. Panel b) shows a three-dimensional volume rendering of a female tract viewed laterally, created by combining multiple two-dimensional slices (note that a 3d slice has been used to virtually cut the tract in half). In both cases brightness represents the degree of tissue staining.

Figure 1
1192x458mm (96 x 96 DPI)





Female reproductive tract volume



<http://mc.manuscriptcentral.com/prsb>
Male ventral spine length