

Sexual conflict drives micro- and macroevolution of sexual dimorphism in immunity

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24 **Abstract**

25 Sexual dimorphism in immunity is believed to reflect sex-differences in trade-offs between
26 competing life history demands. Sexual selection can have major effects on mating rates and
27 sex-specific costs of mating and may thereby influence sex-differences in immunity as well as
28 associated host-pathogen dynamics. Yet, experimental data linking the mating system to
29 evolved sexual dimorphism in immunity are scarce and the direct effects of mating rate on
30 immunity are not well established. Here, we use transcriptomic analyses, experimental
31 evolution and phylogenetic comparative methods to study the association between the
32 mating system and sexual dimorphism in immunity in seed beetles, where mating causes
33 internal injuries in females. We demonstrate that female phenoloxidase (PO) activity, involved
34 in wound healing and defence against parasitic infections, is elevated relative to males as a
35 result of sex-biased expression of genes in the proPO activating cascade. We document
36 substantial phenotypic plasticity in female PO activity in response to mating and show that
37 experimental evolution under enforced monogamy (relative to natural polygamy) rapidly
38 decreases female (but not male) PO activity. The evolution of decreased PO in monogamous
39 females was accompanied by increased tolerance to bacterial infection unrelated to mating.
40 This implies that female responses to costly mating may trade off with other aspects of
41 immune defence. Finally, female (but not male) PO activity show correlated evolution with
42 the perceived harmfulness of male genitalia across 12 species of seed beetles, suggesting that
43 sexual conflict has a significant influence on sexual dimorphisms in immunity in this group of
44 insects. Our results thus provide a proximate and ultimate understanding of the links between
45 sexual selection and sexual dimorphism in immunity.

46

47 **Introduction**

48 Sex differences in immunity are widespread across animal taxa (1–4) and are believed to
49 reflect sex-specific selection and sexually dimorphic life histories (5–9). Sexual dimorphism in
50 immunity may have important consequences both for sex-specific rates of reproduction and
51 survival, with potential impact on population demography (10–13), and for the spread of
52 pathogens. For example, distinct male and female immune systems present more diverse host
53 targets (1,14) and this may influence both disease transmission, infection rates and pathogen
54 virulence (5,12,13,15–20).

55

56 Investment in immune defence is costly. These costs have most often been observed as
57 reductions in fecundity, effectively translating into reproduction-survival trade-offs in the
58 presence of pathogens (9,16,21,22). In polygamous species where sexual selection on males
59 is intense, females are predicted to gain more than males from investing in survival and
60 longevity at the cost of current reproduction and mating effort (3,9,23), and should thus invest
61 more in immunity than males (but see: (2,18,23,24)). Sexual selection may also have
62 pronounced direct effects on optimal investment in immunity, as it may dictate the economics
63 of reproduction (25,26) and lead to elevated mating rates (27), which in turn may increase
64 disease transmission (12,18,19). Therefore, it has been suggested that sexual dimorphism in
65 immunity should increase with sex-differences in optimal mating rates and the strength of
66 sexual selection in males (15,28).

67

68 The effects of sexual selection on sex-differences in immune investment may be magnified in
69 systems where mating is harmful for females, through costs such as the transfer of pathogens
70 during mating, transfer of immunosuppressive seminal fluid substances or direct physical

71 injury (17,29–31). Such male-imposed mating costs are believed to often be results of sexual
72 conflict driven by the different evolutionary interests of the sexes (6–8,32), in which male
73 adaptations evolve to increase reproductive success in competition with other males despite
74 impairing the health of their female mating partners. Females, in turn, evolve counter-
75 adaptations to alleviate the harm inflicted by males resulting in a coevolutionary arms race
76 between the sexes (17,29,32,33). Female immunity responses may represent one such
77 counter-adaptation (34). This suggests that infections or harm on females, induced by sexually
78 selected male mating strategies, may be a significant selection pressure on female immunity
79 in polyandrous taxa (15,18,28), and hence, that the evolution of sexual dimorphism in
80 immunity may in part be an indirect result of male-imposed costs of mating in females.

81

82 Yet, whether and how sexual selection and mating *per se* affect tissue-specific and general
83 immunity is not well understood (5,16,17,30,35). It has, for example, been suggested that
84 tissue-specific (i.e. in the reproductive tract) immune responses upon mating can lead to
85 allocation trade-offs with systemic immunity (30,36). However, few studies have provided
86 direct experimental evidence for a causal link between the mating system and the evolution
87 of sex- and tissue-specific immunity (2,37). To fill this empirical void, we assessed how
88 variation in the intensity of sexual conflict and mating rates in the seed beetle *Callosobruchus*
89 *maculatus* affects i) the evolution of sexual dimorphism in phenoloxidase (PO) activity, a major
90 component of invertebrate immunity involved in wound healing and encapsulation of
91 pathogens, and ii) associated immunopathological consequences of bacterial infections
92 unrelated to mating.

93

94 Sexual selection is intense in *C. maculatus*, including both pre- and post-copulatory processes
95 (38–43), leading to sexual conflict over optimal mating rate and to male traits that cause harm
96 in females during mating (41,42,44,45). The male genitalia carry spines and males with longer
97 spines have greater fertilization success but the spines cause internal injuries in females during
98 mating, leaving females with melanized scars in the reproductive tract (41,42,44). Injurious
99 copulations are wide-spread in insects and may serve several functions, with the ultimate aim
100 to increase male competitive fertilization success (46,47). This may select for increased
101 immune defence locally in the female reproductive tract to enable efficient wound healing
102 and limit female susceptibility to sexually transmitted pathogens (48). Here, we show that PO
103 activity in *C. maculatus* females is high (see also: (34)) and responds dynamically to mating,
104 while it is very low in males. Experimental removal of sexual selection and conflict led to rapid
105 laboratory evolution of decreased female investment in PO activity. This reduced investment
106 was accompanied by the evolution of increased female tolerance to bacterial infection
107 unrelated to mating, suggesting a trade-off between female PO responses to harmful mating
108 and tolerance to other infections. The PO response was paralleled at a macroevolutionary
109 scale, signified by correlated evolution between male genital morphology and sexual
110 dimorphism in PO activity across 12 species of seed beetles.

111

112 **Results**

113 ***A hypothesis about sex-specific immunity trade-offs based on sex-biased gene expression in*** 114 ***the prophenoloxidase-activating cascade***

115 The prophenoloxidase (proPO) activating cascade leads to the production of active PO which
116 serves as an important first-line defence in invertebrates against pathogenic bacteria, fungi
117 and viruses by aiding in wound healing and encapsulation of parasitic infections (49–55).

118 However, the production of PO is strictly regulated (56,57) as it is both energetically costly and
119 generates toxic secondary metabolites such as reactive oxygen species (53,58,59), predicting
120 that investment in PO activity could incur costs to other fitness related traits (16,49,53). To
121 build a more informed hypothesis for how sexual selection and conflict may affect PO
122 investment and its consequences for other correlated immunity traits, we first explored sex-
123 biased expression of *C. maculatus* genes mapping to the proPO activating cascade,
124 functionally annotated in the flour beetle *Tenebrio molitor* (Figure 1). The spätzle processing
125 enzyme (SPE) initiates proPO production which ultimately leads to PO activity (Figure 1). We
126 found that the *C. maculatus* orthologs of the SPE and proPO genes are significantly female-
127 biased in expression, both in the head and thorax as well as in the abdomen of virgin adults.
128 Mating increased transcription of proPO in males, but not in females, where proPO expression
129 remained high irrespective of mating status. Instead, SPE-expression, upstream of proPO gene
130 in the cascade, increased in the female abdomen in response to mating (Figure 1, SI Table 1).
131 In accordance with hypothesized female immunity responses to a harmful mating, these
132 results suggest that females invest heavily in PO activity via SPE and proPO. However, SPE also
133 initiates the modification of Spätzle (SPZ) and downstream Toll-regulated antimicrobial
134 peptides (AMPs), which offer inducible immunity to pathogens. This may thus set the stage
135 for a trade-off between PO (encapsulation) and SPZ (AMP-production) (Figure 1).
136 Overactivation of the proPO cascade may lead to the production of toxic secondary
137 metabolites, suggesting that excessive signalling via SPE to produce high levels of both SPZ
138 and PO may come at a cost to overall health (53,58,59). Here, increased production of serine
139 protease inhibitors (serpins, SPs) via the TOLL-pathway exerts negative feedback and control
140 over SPE activation and activity (60). Notably, both the expression of SPZ and the two putative
141 SPs that we identified in *C. maculatus* were significantly male-biased (SI Table 1, Figure 1).

142 These patterns in gene expression thus suggest a functional molecular basis for sex-specific
143 immunity trade-offs between different components in the pro-PO activating cascade, where
144 females are predicted to invest in PO activity in their reproductive tract in response to harmful
145 mating, but at the potential cost of reduced AMP-production and/or toxic side-effects of
146 overactivation of the proPO activating cascade.

147 [FIGURE 1]

148

149 ***Sex-specific regulation of phenoloxidase activity***

150 To test for sex differences in immunity, we measured PO activity in homogenized whole-body
151 samples of male and female larvae, pupa and adults. The three life stages showed significant
152 differences in mass-corrected PO activity averaged across the sexes ($F_{2,33} = 17.7$, $p < 0.001$,
153 Figure 2a). Larvae showed detectable levels of PO activity, as might be expected given that
154 they feed encapsulated within host seeds together with fecal remains (53). Since we could not
155 determine the sex of the larvae, sex-differences in the larval stage can neither be confirmed
156 nor rejected. Neither male nor female pupae showed measurable levels of PO activity,
157 whereupon there was a drastic and female-limited up-regulation in the virgin adults. The
158 observed effect size of sex on PO activity in virgin adults was, Hedges' $g = 2.08$, which is very
159 high relative to what is typical in insects (mean Hedges' $g = 0.55$; see (2)) and for animals in
160 general (mean Hedges' $g = 0.39$; see (2)). Together with the observed sex-bias in expression
161 of candidate genes involved in the proPO activating cascade, these results imply that sex-
162 specific gene expression generates functional sexual dimorphism in PO activity in *C. maculatus*
163 (Figure 2a).

164

165 To further understand the function of the female-bias in adults, we explored phenotypic
166 plasticity in female PO activity in response to mating. We mated females either only on day
167 one of adult life (treatment 100), on day one and two (110), on day one and three (101), or on
168 all days (111) and measured levels of PO activity subsequently on the third day (2h post mating
169 in 101- and 111 females; ca. 24 and 48h post mating in 110- and 100 females, respectively).
170 The differences among the four treatments were substantial ($F_{3,52} = 18.7$, $p < 0.001$,
171 Supplementary Table 2). The PO activity was high in females when some time had elapsed
172 between mating and PO measurement (i.e. 100- and 110-females), while the levels were near
173 zero in females when PO activity was measured directly after mating (i.e. 101- and 111
174 females) (Figure 2b). Hence, PO activity decreases rapidly after mating but was recovered to
175 almost initial levels within 24h post mating. These results accord with the observed female
176 upregulation of SPE in the abdomen in response to mating (upstream of proPO in the cascade;
177 Figure 1) and may also explain why we did not detect a difference in the expression of the
178 proPO gene in females between virgin state and 24h after mating (SI Table 1).

179 [FIGURE 2]

180

181 Using a subset of 25 females of the same population and generation, we performed a
182 subsequent analysis of PO activity in oviposited eggs. This analysis showed that decreases in
183 female PO activity following mating is unlikely to be due to PO investment in offspring, as all
184 five samples of pooled eggs showed very low (undetectable) levels of PO activity. Hence, the
185 regulation of PO in females seems most likely to occur in the reproductive tract in response to
186 mating itself. We found no evidence of a reproduction-immunity trade-off as there was no
187 relationship between the number of eggs laid by the females over the two days of the
188 experiment and their subsequent measure of PO activity (Supplementary Table 2). Although

189 immunity-reproduction trade-offs are readily observed in insects (9,16), PO investment does
190 not always correlate negatively with fecundity (e.g. (61,62)). Moreover, variation in overall
191 phenotypic and genetic condition (63,64), as well as the amount of male harm inflicted on
192 females (65), could have masked a putative trade-off. Alternatively, trade-offs with PO
193 investment could materialize for other life-history traits (9,21,66), and/or other components
194 of immunity (16,49) (see: Figure 1 and further below).

195

196 ***Experimental evolution of phenoloxidase activity under different mating systems***

197 To directly test the hypothesis that sexual selection and conflict over mating is causing the
198 observed sexual dimorphism in immunity in *C. maculatus*, we compared the levels of PO
199 activity in males and females from replicate experimental evolution lines maintained for 27
200 generations under one of three alternative mating regimes; natural **polygamy** (natural
201 selection and sexual selection – multiple mating); enforced **monogamy** (natural selection but
202 excluding sexual selection – single mating); and **male-limited selection** (applying sexual
203 selection on males but relaxing selection on females – multiple mating but female coevolution
204 prevented). The lines are further described in the *Methods* section and in (45,67). We
205 predicted that females from polygamous lines that had evolved under frequent multiple
206 mating would invest more in PO activity than females from monogamous lines, while the
207 male-limited lines present a control to reveal the extent to which female PO activity may
208 change in the polygamous mating system via genetic correlation when selection acts only via
209 males. We also tested whether the direct effect of mating and reproduction on PO activity had
210 evolved under the different mating systems by for all lines comparing the PO activity of virgin
211 and socially naïve individuals to that of beetles allowed to mate and reproduce in groups of 5
212 males and 5 females prior to the PO measurements.

213

214 We analysed the effects of experimental evolution regime crossed by mating treatment in
215 Bayesian mixed effect models using the MCMCglmm package (68) for R (69). Experimental
216 evolution line replicates, crossed with mating treatment, were included as random effects
217 (priors and model specification in Supplementary 3).

218

219 The mating treatment decreased body mass relative to the virgin treatment, revealing a
220 sizeable investment in reproduction by both sexes (SI Table 3a). While males did show an up-
221 regulation of proPO gene expression in response to mating (SI table 1), they did not have any
222 detectable levels of PO activity (SI Table 3c), confirming that PO investment is strongly female-
223 biased in the adult stage in *C. maculatus* (63). In females (N = 358 assays), the mating
224 treatment significantly decreased PO activity ($\Delta PO = -0.029$ (-0.022; -0.037), $P_{MCMC} < 0.001$)
225 but this effect was similar in the three selection regimes (all pairwise interactions $P_{MCMC} > 0.6$)
226 (Figure 3). Importantly, evolution without sexual conflict under the monogamy regime had led
227 to a general decrease in female PO activity relative to the polygamy regime ($\Delta PO = -0.010$ (-
228 0.002; -0.018), $P_{MCMC} = 0.030$), confirming a key prediction. The monogamy regime also
229 showed lower levels of PO activity compared to the male-limited regime, where females had
230 been kept under relaxed selection ($\Delta PO = -0.011$ (-0.004; -0.020), $P_{MCMC} = 0.012$). Accordingly,
231 the polygamy and male-limited regime had similar levels of PO activity ($P_{MCMC} > 0.8$, Figure 3).
232 Thus, when the expected number of matings decreased to a single mating in the monogamy
233 regime, the optimal female strategy was to decrease PO activity, in support of the hypothesis
234 that PO investment is costly and likely trades off against other female fitness components
235 (16,49,53,70). If immune defence is costly, a corollary from allocation theory is that
236 polygamous females should invest in PO in relation to their total energy reserves and expected

237 number of partners. In contrast, among monogamous females we expect the evolution of
238 decreased condition dependence due to their overall lower PO investment. This is also what
239 we find; there was a positive relationship between female body mass and PO activity in
240 polygamous lines (slope = 0.011 (0.005; 0.016), $P_{\text{MCMC}} < 0.001$), whereas this relationship was
241 absent in monogamous lines ($P_{\text{MCMC}} = 0.48$), and this regime-difference in the condition
242 dependence of PO investment was significant ($\Delta\text{slope} = 0.007$ (0.001; 0.013), $P_{\text{MCMC}} = 0.026$,
243 Figure 3).

244 [FIGURE 3]

245

246 Again, however, a fecundity cost of high PO activity was not apparent when comparing
247 regimes; offspring production in the reproducing treatment was higher for females from the
248 polygamy regime (showing higher levels of PO activity) than for monogamous females (with
249 lower levels of PO activity) (SI Table 3b).

250

251 ***Experimental evolution of the response to bacterial infection***

252 To explore possible consequences of increased female allocation to PO activity in response to
253 mating (Figure 1), we measured survival in the monogamy and polygamy lines when exposed
254 to bacterial infection in abdominal tissue adjacent to the reproductive tract. Females (total n
255 = 1060, 24-48h past adult eclosion) were either virgin or mated prior to being infected with
256 one of two doses (OD1 or OD2) of the entomopathogenic gram positive bacteria, *Bacillus*
257 *thuringiensis*, or a sham control (pricking with a sterilized needle dipped in PBS buffer). We
258 analysed survival in mixed effects Cox proportional hazard models using the coxme package
259 (71) for R, with regime and mating treatment as fixed effects and replicate lines as random
260 effects. We also confirmed results by using the MCMCglmm package (68) to apply Bayesian

261 mixed effect models on a binomial response variable (dead/alive on day 5 post infection),
262 which allowed us to add fully crossed random effects (line by treatment) in the analysis (Full
263 statistical summaries in Supplementary Tables 4a, b).

264

265 Females from the monogamy regime, that had evolved lower levels of PO activity, showed
266 higher survival under bacterial infection (treatment:regime interaction: $X_2^2 = 13.7$, $P = 0.001$,
267 Figure 4a-d). This suggests that the evolution of female allocation to PO in response to
268 expected harmful mating incurs costs for general susceptibility to infection. Mating by itself
269 led to an increase in mortality ($X_1^2 = 63.6$, $P < 0.001$), likely due to a shift in allocation from
270 survival to reproduction in mated females. However, there was no significant effect of either
271 mating status (treatment:mating interaction: $X_2^2 = 1.2$, $P = 0.56$) or the interaction between
272 evolution regime and mating status (three-way interaction: $X_2^2 = 0.14$, $P = 0.93$, Figure 4a-d).
273 Although somewhat surprising, this result is not inconsistent with a trade-off between female
274 PO investment in the reproductive tract and vulnerability to systemic infection caused by
275 other pathogens, as also virgin females display high PO activity prior to being mated (SI Table
276 1, Figure 2), and females from the polygamy regime do so more than females from the
277 monogamy regime (Figure 3). The hypothesis that female investment in PO entails costs in
278 terms of increased vulnerability to other infections was further supported by the fact that
279 virgin males from monogamous and polygamous regimes (which do not seem to invest in PO
280 at all) did not show any strong differences in their response to bacterial infection (assessed in
281 a separate experiment, $X_2^2 = 0.94$, $P = 0.63$, Figure 4e,f). However, the sample size in the male
282 experiment was smaller ($n = 270$ for virgin males compared to $n = 493$ for virgin females),
283 limiting direct comparisons between the male and female assays. Nevertheless, the male

284 experiment did reveal an overall effect of the bacterial injection ($X^2_2 = 7.77$, $P = 0.021$) and
285 significantly greater survival of polygamous males ($X^2_2 = 6.63$, $P = 0.010$) (SI Table 4c).

286

287 To gauge the generality of these results, and to further investigate whether the higher survival
288 of monogamous females under bacterial infection was due to more efficient clearing of the
289 bacterial infection (greater resistance), or because they were better at withstanding it (greater
290 tolerance) (72), we infected once-mated polygamous and monogamous females with the
291 gram-negative bacteria *Pseudomonas entomophila* using the same protocol as described
292 above. The *P. entomophila* strain used is resistant to the antibiotic ampicillin. This allowed us
293 to screen a subset of females collected 12h post infection exclusively for *P. entomophila* by
294 culturing female cell tissue on Luria agar plates with ampicillin. Again, females from the
295 polygamy regime showed higher susceptibility to bacterial infection (treatment:regime
296 interaction: $X^2_2 = 16.6$, $P < 0.001$, total $n = 288$, Figure 4g,h, SI Tables 4d,e). However, there
297 was no significant difference in bacterial load among the evolution regimes ($P_{\text{MCMC}} > 0.2$, n
298 samples = 63, n females = 189, Figure 4i, SI Table 4f), suggesting no large differences in the
299 ability of females to clear the bacterial infection, but rather that monogamous females were
300 more tolerant to infection. This result suggests that the reason why polygamous females suffer
301 increased mortality costs under infection may not necessarily be directly due to an allocation
302 trade-off between the production of PO and AMPs (Figure 1), but to toxic secondary
303 metabolites resulting from overexpression of the proPO activating cascade (53,57). More
304 generally, the results are consistent with the hypothesis that sexual conflict and harmful
305 mating can lead to increased vulnerability to infection in females as a result of sex-specific
306 trade-offs between different components of immunity (17,30,36).

307 [FIGURE 4]

308

309

310 ***Correlated evolution between female PO activity and male genital morphology***

311 We explored whether macroevolutionary transitions in sexual dimorphism in immunity could
312 be driven by the evolution of mating interactions and the harmful morphology of male
313 genitalia in this group of insects (23). We measured PO activity in virgin males and females of
314 12 species of seed beetles. There was pronounced sexual dimorphism and female-limited
315 expression in many species (SI Figure 5a). To quantify harmfulness of the male genitalia in
316 each species, we asked two expert and ten naïve biologists to rate pictures of male genitalia
317 for the perceived harm they cause in the female reproductive tract (SI Figure 5b). Importantly,
318 earlier work has shown that male harm assayed in this manner correlates positively with the
319 amount of scarring that occurs in the female copulatory tract after mating (23). Species
320 differences explained 61% of the total variation in rater scores and scores were highly
321 correlated between experienced and naïve raters ($r = 0.83$), suggesting that raters generally
322 agreed on the classification of male harm. Female and male PO activity, as well as male harm,
323 showed moderate phylogenetic signals (Blomberg's $K = 0.68, 0.52$ and 0.54 , respectively (73)).
324 Hence, we applied a phylogenetic generalized least squares regression (PGLS) based on
325 species means using the ape package (74) for R, accounting for phylogenetic dependencies
326 using Orlin-Uhlenbeck estimation and an extant seed beetle phylogeny (75,76). There was
327 significant positive covariance between male harm and female PO activity ($\alpha = 6.70$,
328 standardized slope = 0.83 , $df_{12,10}$, $P < 0.001$, SI Table 5a). Moreover, the covariance between
329 male harm and male PO activity was not significant and opposite in sign ($\alpha = 2.92$, standardized
330 slope = -0.57 , $df_{12,10}$, $P = 0.08$, SI Table 5b). These analyses confirm our experimental findings

331 and show that sexual conflict is apparently capable of driving macro-evolutionary divergence
332 in sexual dimorphism in immunity (Figure 5).

333 [FIGURE 5]

334

335 **Discussion**

336 Sexual selection can result in increased male harm to females during mating (22,29,32), either
337 through direct injury or infection with pathogens, and this should in theory favour increased
338 female investment in immunity when female lifetime reproductive success is elevated by
339 increased longevity (16,18,23,26). Here, we provide a suite of experimental and comparative
340 data collectively showing that sex-differences in immunity can be modulated by sexual conflict
341 in a species where costs of mating are conspicuous. This conclusion is based upon
342 observations of (1) sex-biased gene expression in the proPO activating cascade (Figure 1), (2)
343 a functional female-bias in PO activity which is substantially higher than what is typical in
344 insects, (3) female-limited phenotypic plasticity in PO activity in response to mating (Figure 2),
345 (4) female-limited microevolutionary changes in immunity traits in response to experimental
346 manipulation of the mating system and hence sexual conflict (Figures 3 and 4), and (5)
347 correlated evolution between male genital morphology and female PO activity across species
348 (Figure 5).

349

350 Few studies have quantified female immune responses post mating (5,17) and it often remains
351 unclear whether male harm via genitalia or ejaculatory compounds (i.e. sexual antagonism)
352 drive such responses, or whether they represent independent female optimization of the
353 trade-off between current and future reproduction. Here, we directly manipulated the level
354 of sexual selection and conflict, which is relatively well understood in *C. maculatus* (e.g.

355 (33,34,41,44,77–80)), and found a clear female-limited PO response, while no correlation
356 between female reproductive investment and PO activity was detected. Hence, our data point
357 to male harm inflicted during mating as the driver of female PO investment. In this system,
358 the inflicted harm by a male on his female mating partner is positively correlated to his success
359 in sperm competition (29), presumably because seminal fluid substances (66) that benefit
360 males in sperm competition (62) pass more rapidly into the female body if the copulatory duct
361 is ruptured (32). However, these wounds may leave females at a risk of systemic infection with
362 pathogens (36), suggesting a need for healing these injuries via a PO mediated, potentially
363 costly, reaction.

364

365 We hypothesized that these effects could have consequences for female susceptibility to
366 infections unrelated to mating via trade-offs between PO activity and other components of
367 immunity in the prophenoloxidase activating cascade (36) (Figure 1). This prediction was
368 supported by the increased susceptibility to bacterial infection observed in females from the
369 polygamous mating regime (Figure 4). It is possible that the need for high PO activity in the
370 reproductive tract of polygamous females led to a harmful “overactivation” of the proPO
371 activating cascade upon bacterial infection in adjacent abdominal tissue, as the same
372 proteolytic cascade is involved in the production of both proPO and AMPs needed to fight
373 bacterial infections (e.g. (81)). While such overactivation could mask allocation trade-offs by
374 attending the dual need of producing PO and AMPs, it may have caused an inflammatory
375 response with increased mortality of polygamous females as a result. Indeed, the proPO
376 activating cascade can have detrimental immunopathological consequences via the
377 production of toxic secondary metabolites and needs to be strictly regulated (59), and severe
378 bacterial infection can kill the organism also via side-effects of excessive melanization (53,58).

379 Further efforts are needed to test this possibility and to pin-point the exact causality behind
380 the observed effect of mating system on the female-limited correlated evolution of increased
381 PO investment and decreased tolerance to bacterial infection.

382

383 Male reproductive success in polyandrous mating systems is typically maximized by a shift
384 towards current reproduction in the female mating partner, as this would increase the
385 likelihood of the male siring a larger fraction of the offspring produced by the female
386 (12,17,18,30). This predicts that males should evolve to manipulate females to invest in
387 current reproduction at the expense of reduced immunity and longevity. In line with these
388 predictions, males with longer genital spines, that inflict more harm during mating, sire more
389 offspring in *C. maculatus* (41,44) and seem to stimulate female fecundity (*unpublished data*).
390 Moreover, the male ejaculate regulates female immunity post mating in *Drosophila*, guppies,
391 mice and humans (30,31,35,36,82,83), although it often remains unclear to what extent the
392 effects are detrimental or beneficial to the female overall. It has even been suggested that
393 males may gain fitness benefits by transferring sexually transmitted diseases that trigger shifts
394 in female allocation towards current reproduction (15,84), but this possibility lacks empirical
395 support (85). In other insects, female PO activity either increases or decreases post mating
396 and it has been suggested that in species where mating downregulates female PO activity,
397 males corrupt the female immune function (17). While our results are not inconsistent with
398 this hypothesis, they rather suggest that *C. maculatus* females are “primed” for harmful
399 mating and that PO activity in females initially decreases post mating as a result of wound
400 healing but is quickly restored. Similar female anticipatory immunity activation has also been
401 observed in *Drosophila* (86,87).

402

403 When mating rate affects both sexual dimorphism in immunity and infection rates, this can
404 result in intricate eco-evolutionary dynamics with demographic consequences for both host
405 and pathogen (5,12,15–19). Our study suggests that sexual conflict over mating rate can drive
406 sexual dimorphism in immunity and that allocation to different components of immunity may
407 play an important role in mediating effects of mating on immunopathological responses in
408 females. In *Drosophila*, mating increases immune response in reproductive tissue, and in most
409 insects mating decreases general immunity, but causality typically remains unclear (16). Our
410 results imply that baseline PO activity decreases in *C. maculatus* females as a genetic response
411 to the alleviation of sexual conflict and harmful mating. Moreover, monogamous females, that
412 evolved a reduced investment in PO activity relative to naturally polygamous females, showed
413 an associated evolutionary increase in tolerance to bacterial infection in abdominal tissue
414 adjacent to the reproductive tract, effects not seen in their conspecific males. This suggests
415 that sex-specific trade-offs determine the mosaic of immune investment and that sexual
416 selection and conflict affect the economics of these trade-offs. This complexity may explain
417 some of the discrepancies found in the literature concerning female immune responses to
418 mating (reviewed in: (5,17)) and motivates further explorations of the selection pressures
419 affecting sexual dimorphism in immunity.

420

421

422 **Methods**

423 ***Study populations***

424 *Callosobruchus maculatus* females lay eggs on seeds and larvae burrow into the seed where
425 the entire development occurs. Beetles emerging from seeds are reproductively mature and
426 require neither water nor food to reproduce successfully (e.g. (88,89)). Adults typically die 7-

427 14 days after emergence in the absence of food or water (e.g.(90). All experiments used
428 beetles originating from a genetic stock that was originally sampled in Lome, Togo, in 2010,
429 and subsequently maintained as 41 isofemale lines in the laboratory to maintain the genetic
430 variation present in the original population (91), before being mixed into a large, outbred, and
431 genetically diverse experimental population ($N \sim 500$). This genetic stock has been used in
432 quantitative genetic designs (e.g. (77,91–93), artificial selection experiments (94), and
433 experimental evolution (45,67,95) to demonstrate substantial sex-specific standing genetic
434 variation in behavior, morphology, life-history and life time reproductive success, as expected
435 given that the lines originate from the center of the species range (96).

436

437 The experimental evolution lines used to study the effect of the mating system on the
438 evolution of sexual dimorphism in immunity are thoroughly described in (45,67). In brief, the
439 lines were maintained under standard temperature (29°C), humidity (50%RH) and light cycle
440 (12L: 12D), and were reared on the preferred host plant (96) *Vigna unguiculata* (black-eyed
441 bean). There are three replicate “Monogamy” lines, three “Polygamy” lines and two replicate
442 “Male-limited” lines. Effective population size for the lines in each regime was kept
443 approximately equal ($N_e \approx 150$; $N_{\text{Male-limited}} = 200$, $N_{\text{Monogamy}} = 246$, $N_{\text{Polygamy}} = 300$) and the
444 number of beans provided as egg laying substrate in each regime was standardized to give the
445 same, relatively low, juvenile density (2-4 eggs/bean) to minimize (and equalize) larval
446 competition (45). To implement the different regimes, selection was only applied for the first
447 two days of adult life. However, the reproductive output over these first days typically
448 corresponds to half of the total lifetime reproductive output. The regimes show differences
449 consistent with generally positive effects of sexual selection on genetic quality in terms of

450 increased female reproductive success and population productivity in polygamy lines relative
451 to monogamy lines at generations 16 and 20, respectively (45). They also show differences in
452 sexually selected male pre- and post-copulatory traits (67,95).

453

454 ***Expression of genes involved in the proPO activating cascade***

455 To assay the effects of sex and mating status on the expression of relevant genes, we used
456 data previously published in (97). Briefly, RNA sequencing (Illumina TruSeq) was used to test
457 for sex differences in gene expression in virgin and mated age-matched beetles, separately for
458 reproductive and non-reproductive tissues (i.e. abdomen and head & thorax, respectively). In
459 the mating treatment, RNA was extracted 24h after mating. We pooled six individuals of each
460 sex, tissue and treatment and replicated these pools three times. The transcriptome was
461 assembled *de novo* (98), and differential expression analysed using edgeR, as described in(97).
462 The candidate PO genes were detected using BLAST (tblastn search in the TSA database for *C.*
463 *maculatus*, using the protein sequences as query) and here we report the ones with a
464 significant sex difference in expression (with a false discovery rate adjusted p-value < 5%) in
465 the virgin beetles in either tissue category. To generate hypotheses about sex-specific
466 immunity trade-offs, we mapped the general sex-differences onto the pro-PO cascade as
467 functionally annotated for the flour beetle *Tenebrio molitor* (55,60) (Figure 1).

468

469 ***Phenoloxidase assays***

470 Individual beetles were homogenized by 20 seconds of grinding with a pestle in an Eppendorf
471 tube containing 20 µl Phosphate Buffered Saline (PBS). Samples were kept on ice until
472 centrifuged at 17g for 10 min at 0°C, and the supernatants (10 µl) were stored at -80°C prior
473 to the assay of PO activity. The frozen homogenates were analysed by an investigator

474 uninformed of the samples' identity and treatment affiliation, i.e as blind tests. Due to the
475 small volume of each sample and high background due to the crude protein extract, the assay
476 was first developed and optimized to ensure that proper enzyme kinetics were at hand. In
477 preliminary experiments the beetle homogenate was preincubated with curdlan (a β -1,3-
478 glucan), trypsin or chymotrypsin to fully convert all zymogenic proPO to the active enzyme PO
479 before assay of enzyme activity. However, the frozen homogenates did not show any
480 increased PO activity after activation, indicating that the preparation method such as freezing
481 at -80 C had converted all proPO into active enzyme PO. Dopamine, L-Dopa and 4-
482 methylcatechol+hydroxyproline ethyl ester were each tested as substrate for *Callosobruchus*
483 PO, and dopamine was shown to be the most efficient substrate and was used in the further
484 experiments, and phenylthiourea could completely block the activity (data not shown). For
485 the experimental samples, six samples of beetle homogenate at a time were randomly chosen
486 and thawed. After thawing, each individual beetle homogenate (3 μ l) was incubated together
487 with 7 μ l PBS and 50 μ l dopamine [10 mM in H₂O] at 22°C. The reaction proceeded for 15
488 minutes after which 60 μ l H₂O was added to terminate the reaction and after centrifugation
489 at 16000 x g for 1 min the absorbance at 420 nm was recorded. For each sample, a blank
490 control was assayed containing 3 μ l beetle homogenate, 7 μ l PBS and 50 μ l H₂O, and was
491 incubated and measured as the samples above. The enzyme activity is expressed as increase
492 in absorbance at 420 nm per minute in the focal sample relative to its blank control
493 ($\Delta A_{420}/\text{min}$).

494

495 ***Sex-specific ontogenetic regulation of phenoloxidase activity***

496 The eggs laid by the females in the mating status experiment (below) were followed through
497 ontogeny. We sampled a total of 20 final instar larvae, 20 pupae and 14 adults. Larvae of *C.*

498 *maculatus* could not be sexed. Pupae were sexed by abdominal morphology, for a total of 10
499 male and 10 female pupae. Virgin adults were collected as virgins within 0-36 hours post
500 emergence. All individuals were weighed and measured for PO activity. We analysed
501 differences between developmental stages by adding mass of the tissue analysed as a
502 covariate in an ANCOVA. As we could not determine the sex of larvae, we performed one
503 model that averaged effects across the sexes and one model where we excluded larvae and
504 could retain sex. Both models showed significant differences between life stages.

505

506 ***Female phenotypic plasticity in phenoloxidase activity in response to mating.***

507 We used males and females from the Lome base population, reared at standard conditions.
508 All adults were virgin and between 24-48 hours old at the start of the experiment. On day one,
509 120 females were individually placed in small 30mm diameter petri dishes together with two
510 males, in three separate bouts (40 females at a time). Matings were observed and mated
511 females were immediately removed and placed into a 90mm diameter petri dish containing
512 black eyed beans allowing females to oviposit. In total, 114 of the 120 females mated
513 successfully over an observation period of 20 minutes per bout. A random set of 35 of these
514 females were assigned to treatment 100 (mating on day one and then reproduction in
515 isolation until being measured for PO activity on day three). The rest of the females were given
516 the opportunity to mate on day two and day three, but all females did not mate on all days.
517 This resulted in four treatment groups; 100, (mated on day 1 only), 110 (mated on day 1 & 2),
518 101 (mated on day 1 & 3) and 111 (mated on all days). Approximately two hours after the final
519 mating on day three, all females were weighed and then measured for PO activity as described
520 above. Measuring PO activity is time-consuming, and since preliminary analyses of the first
521 batch of females suggested sufficient power to detect effects of mating status (see Figure 2),

522 all females were not measured. The following sample sizes were attained for each treatment;
523 100: 15, 110: 7, 101: 13, and 111: 23 females. We also counted the number of adult offspring
524 produced by each female over the 48h of egg laying. We analysed the effect of mating status
525 and number of offspring produced, including their interaction, on female PO activity in an
526 ANCOVA. Female body mass at the time of homogenization was included as a covariate.

527

528 To determine whether female PO is allocated to eggs, 10 matured eggs per female were
529 dissected out from 25 virgin females for a total of five samples containing 50 eggs each
530 (corresponding to approximately 50% of the lifetime production of eggs of a single female).
531 Samples were weighed and then subjected to the same crushing and centrifuging protocol as
532 the mated females before being frozen at -80 °C and later measured for PO activity.

533

534 ***Experimental evolution of phenoloxidase activity under alternative mating regimes***

535 The experiment was performed following 27 generations of experimental evolution and one
536 subsequent generation of common garden (polygamy) selection through standard culturing
537 to remove any potential influence of parental environmental effects. PO activity was
538 measured in the whole body of single male and female beetles from two replicate lines from
539 each mating regime (6 lines in total). To manipulate the reproductive status of the beetles,
540 newly emerged virgin adults (0-48h old) were either placed together in 90mm diameter petri-
541 dishes in groups of five males and five females that were allowed to reproduce (“Reproducing”
542 treatment), or in petri dishes with 5 males and 5 females but individually isolated in aerated
543 Eppendorf tubes (“Virgin” treatment). All petri dishes contained black eyed beans, so that all
544 beetles experienced the olfactory stimuli of the host beans, but only reproducing females
545 could oviposit on the beans. After 46h, individuals were weighed before being put through

546 the protocol to measure PO activity (see above). Beans from the mating treatment were
547 stored until adult offspring emerged. Offspring were frozen and -20°C and later counted to
548 estimate allocation to reproduction in all regimes. We set up the experiment in two separate
549 batches one week apart in time, with each batch containing one replicate line of each
550 evolution regime. We analysed differences among evolution regimes and mating treatments
551 in Bayesian mixed effect models implementing Markov chain Monte Carlo simulations using
552 the MCMCglmm package (68) in R (69). We ran separate models for males and females as PO
553 activity was virtually undetectable in males. Evolution regime and mating treatment, including
554 their interaction, were added as fixed effects and body mass was added as a covariate to
555 control for the amount of tissue analysed as we used whole-body samples. In follow-up
556 analyses we also assessed interactions between female body mass and the other two fixed
557 effects (to test for condition-dependence of PO activity; see Results). Non-significant fixed-
558 effect interactions were removed sequentially if $P > 0.2$. We blocked out effects of batch by
559 adding it as a fixed effect. Similarly, we also blocked out the potential effect of freezing some
560 individuals before homogenizing samples, something that had to be done for logistic reasons.
561 Replicate line crossed with mating treatment, and adult mass when appropriate, were always
562 included as random effects when estimating effects of evolution regime on PO activity. We
563 used weak and unbiased priors for the random effects and ran models for 3,000,000 iterations,
564 preceded by 100,000 burn-in iterations that were discarded, and stored every 3,000th
565 iteration (thinning), resulting in 1,000 uncorrelated posterior estimates of the fixed effects
566 upon which we calculated Bayesian P-values and 95% credible intervals. Prior specification
567 and MCMC settings were the same for all models (exemplified in Supplementary Table 3c).

568

569 ***Evolution of the response to bacterial infection***

570 At generation 50, we collected beetles from each of the three replicate populations of the
571 Monogamy and Polygamy regime and then maintained them under common garden
572 conditions (natural polygamy) for one generation to minimize environmental parental effects.
573 To measure evolved vulnerability to a bacterial pathogen, we first isolated 2-day-old
574 experimental virgin females from each of the lines and paired them individually with a single
575 male from their own line for 5 hours. Simultaneously, we also collected another subset of
576 females that were held as virgin throughout the experiment. On day three post eclosion, we
577 infected females with a strain of the entomopathogenic gram-positive bacteria *Bacillus*
578 *thuringiensis*, described in (99). Beetles were first anesthetized with carbon-dioxide and then
579 pricked at the lateral side of the lower abdomen, using a 0.1mm minuten pin (Fine Science
580 Tools) dipped in overnight bacterial suspension of 1 OD or 2 OD (subcultured from an
581 overnight culture of the bacteria). We performed sham infection with a pin dipped in sterile
582 PBS solution. Following the infection (or sham infection), we isolated females individually in
583 24 well-plates. We monitored individual survival at every 12 hours until 48 hours post
584 infection and daily around 6pm for the next 8 days. Females still alive 10 days post infection
585 (less than 30%) were right-censored in the subsequent survival-analysis. In a separate
586 experiment, we also measured post-infection survival of 3-day old virgin males as described
587 above.

588 At generation 54, we again collected mated females from two randomly selected replicate
589 populations each of Polygamy and Monogamy and maintained them under common garden
590 conditions. In the subsequent generation (Gen 55) we collected virgin females from each
591 regime. We first mated two-day old females with a male from their own population. We then
592 infected the females with a 0.5OD or 1.0OD solution of the gram-negative bacteria
593 *Pseudomonas entomophila* using the same protocol as described above. Following infection,

594 we housed females individually in the 24 well plates. Survival was first observed after 12
595 hours and a subset of beetles were taken out for bacterial load assay described below. We
596 measured survival up to 120 hours post infection.

597 The *P. entomophila* strain used is resistant to the antibiotic ampicillin. This allowed us to
598 screen the females collected 12h post infection exclusively for *P. entomophila* by culturing
599 female cell tissue on Luria agar plates with ampicillin (0.1mg/ml) and subsequently counting
600 bacterial cultures on the plates to estimate bacterial load. We first collected 3 surviving
601 females 12hours post infection and transferred them to a micro-centrifuge tube. We then
602 washed the three beetles together with 70% ethanol twice. Following the ethanol wash we
603 again washed them with sterile water once. Subsequently, we added 90 μ l of PBS and
604 crushed the beetles together using a sterile micro-pestle. From this master-stock solution we
605 made dilutions up to 10^{-5} in 96 well plates. We spotted 3 μ l of each dilution on Luria agar
606 plates with ampicillin. We kept the plates over night at 27°C and counted distinguishable
607 *Pseudomonas entomophila* colonies. From the number of colonies, we calculated the
608 bacterial load per female beetle and used that for further analyses. In total we calculated
609 load for 8 samples per line and bacterial concentration. One sample was lost, resulting in a
610 total of 63 samples (each based on 3 females). Analyses described in the Results and model
611 specifications in Supplementary 4.

612

613 ***Correlated evolution between PO activity and male genital morphology***

614 We measured the PO activity of 5 virgin males and 5 virgin females of each of the 12 species
615 (see Figure 5) using whole-body samples. All individuals were less than 48h old post adult
616 emergence. As the species differ widely in body size, we modified the amount of PBS buffer

617 added at homogenization to retain more equal concentration of tissue for all species in the
618 original samples to be analysed for PO activity.

619

620 We used a modified version of the protocol of (23) to assess variation in the injuriousness of
621 male genitalia. We first dissected out the male genitalia of 2 individuals per species. Each
622 genitalia was photographed twice from complimentary angles to describe the 3D structure of
623 the aedeagus (the intromittent apical part of male genitalia). This resulted in 48 photos of the
624 24 male samples. The two complimentary photos of each genitalia were placed together on a
625 sheet and given a random ID to hide the species identity for raters. We asked 10 colleagues
626 (evolutionary ecologists at our institution) to individually rate the 24 male genitalia on a scale
627 from 0-10 in terms of the harm they predicted that the genitalia would cause inside the female
628 reproductive tract during mating. Two of the authors of this study, with ample experience of
629 sexual conflict theory and seed beetle biology (GA and JLR) also rated the genitalia (without
630 knowledge of the recorded PO activity in the species, except for *C. maculatus*). The scores of
631 naïve and experienced raters were highly aligned (see: Results), suggesting that the rating of
632 male harmfulness was unbiased in terms of prior knowledge of the mating system. We
633 extracted a mean score for predicted harmfulness for each of the 24 males based on scores
634 from all 12 raters.

635

636 We analysed the covariance between harmfulness of the male genitalia and male and female
637 PO activity based on species means across the phylogeny using phylogenetic least squares
638 (PGLS) regression with Ohrstein-Uhlenbeck correction implemented in the ape package(74)
639 for R (model specification and output in Supplementary Table 4). All variables were variance
640 standardized in the analyses. Given the uncertainty of exact branch lengths, we set all

641 branches to unit length. PO measurements were divided by the concentration of tissue in each
642 sample prior to analysis.

643

644

645 ***Author Contributions***

646 JB and IMA created and maintained the selection lines. QC performed the experiments on PO
647 activity of the selection lines. EP performed experiments on mating status and ontogeny. JLR
648 and EP prepared species samples and JLR dissected male genitalia across species. IS performed
649 all PO activity assays. BB and DBa planned and performed measures of responses to bacterial
650 infection in the evolution lines. EI and AS performed the bioinformatic analyses. DBe analyzed
651 all other data together with JB. DBe planned and conceived the study with considerable input
652 from IS, GA and IK. DB wrote the first draft of the manuscript with input from all authors.

653

654 ***Competing Interests Statement***

655 The authors declare no competing interests

656

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664

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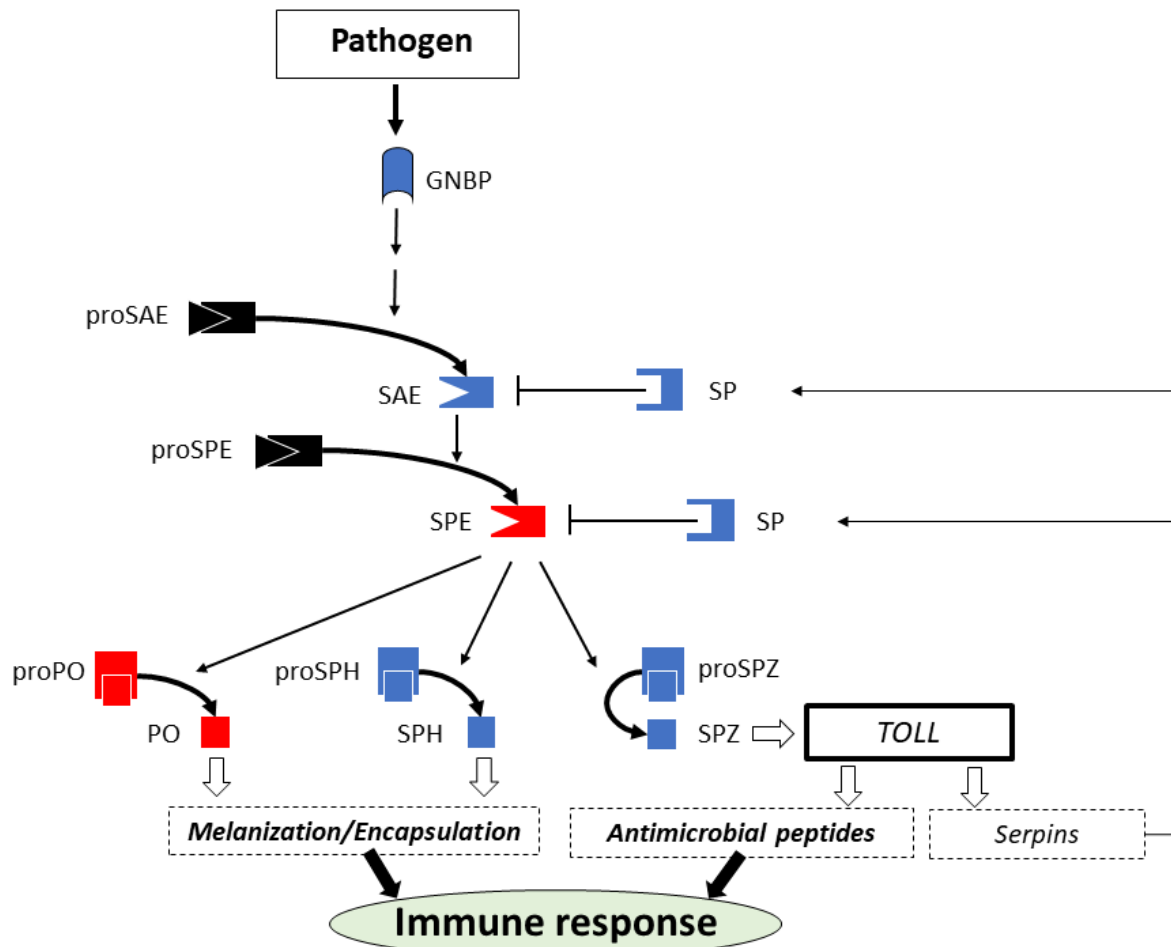
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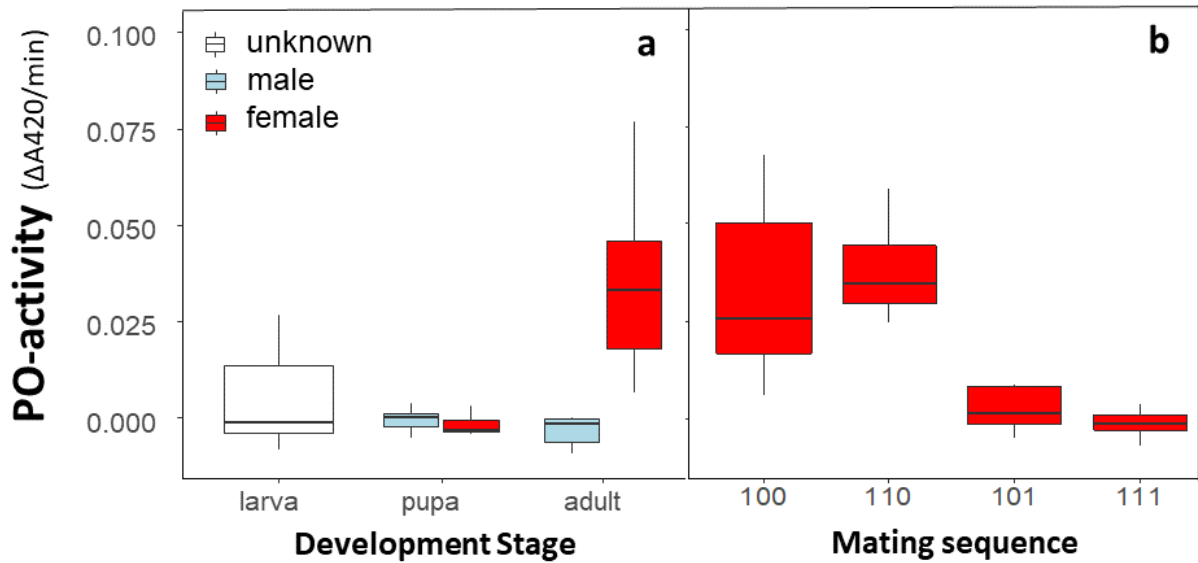
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905 **Figure 1: Sex-biased gene expression in the proPO signaling cascade.**

906 Schematic representation of adult sex-biased gene expression (significant female bias = red,
 907 significant male bias = blue) in the proPO activating cascade, based on previous studies of *Tenebrio*
 908 *molitor* (55,60) parameterized with gene expression data for *C. maculatus* (97). Spätzle processing
 909 enzyme (SPE) initiates proPO and proSPH production (leading to PO and SPH, respectively) which
 910 ultimately leads to encapsulation, melanization and wound healing as a first line of defense against
 911 pathogens. However, SPE also initiates the production of Spätzle protein (SPZ) from proSPZ, which
 912 ultimately leads to increased production of antimicrobial peptides (AMPs) via the TOLL pathway,
 913 which offers inducible immunity against pathogens, thus setting the stage for an allocation trade-off
 914 between proPO/proSPH (encapsulation) and proSPZ (AMP-production). Overactivation of the proPO
 915 cascade has toxic side-effects via the production of secondary metabolites (ROS), suggesting that
 916 overproduction of SPE may come at a cost to overall health. Here, production of serpins in the TOLL-
 917 pathway exert negative feedback and control over SPE-production via Serine protease inhibitors
 918 (SPs). Females show more expression of SPE, leading to downstream female-bias in the expression of
 919 proPO. Males show more upstream signalling and more production of proSPZ, which initiates
 920 production of antimicrobial peptides and regulates the cascade through negative feedback via TOLL.
 921 These patterns in gene expression suggest a mechanistic basis for sex-specific immunity trade-offs
 922 between different components in the pro-PO activating cascade, where females are predicted to
 923 invest more in wound healing in their reproductive tract in response to mating at the potential cost
 924 of reduced AMP-production and/or toxic side-effects of overactivation of the proPO pathway.

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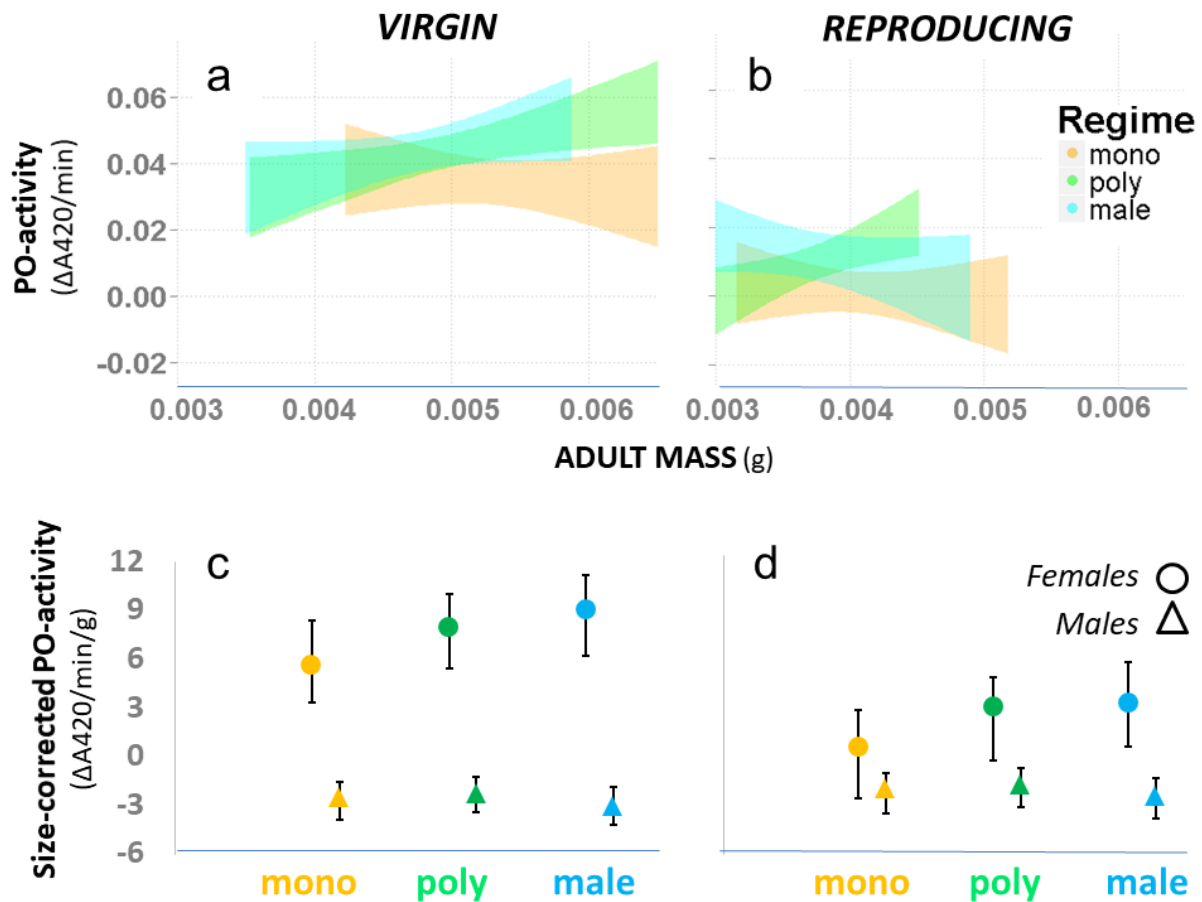
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928 **Figure 2: Sex-specific regulation of phenoloxidase levels.**

929 **(a)** There were significant differences in PO activity throughout development, with levels near zero
930 detected in male (blue) and female (red) pupae and virgin adult males, but detectable levels in
931 (unsexed = white) larvae and very high levels in virgin adult females. **(b)** PO activity measured on day
932 3 in females mated only on day one (100), day one and two (110), day one and three (101), or on all
933 days (111), showed that PO levels are reduced after mating but are restored to initial levels within
934 24h post mating. Shown are medians \pm the first and third quartiles (boxes) and min/max values
935 (whiskers).

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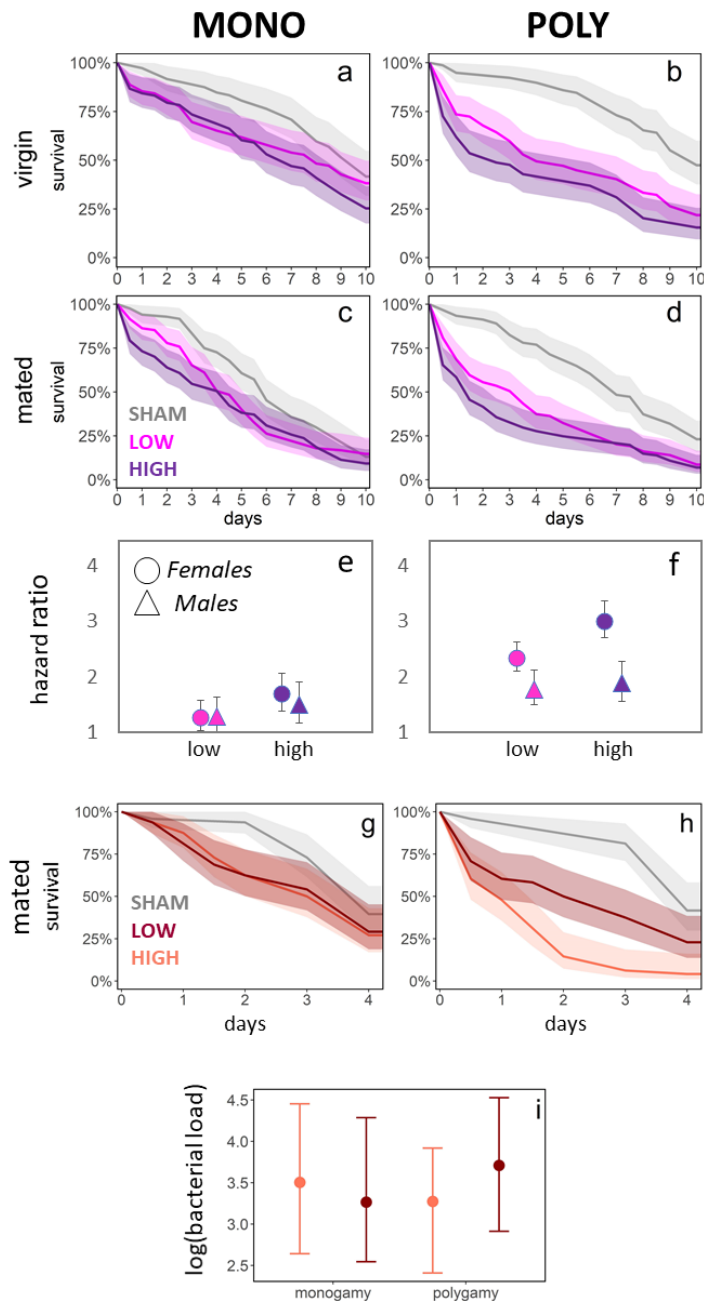
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940 **Figure 3: Microevolutionary change in PO activity during experimental evolution under**
 941 **alternative mating regimes.**

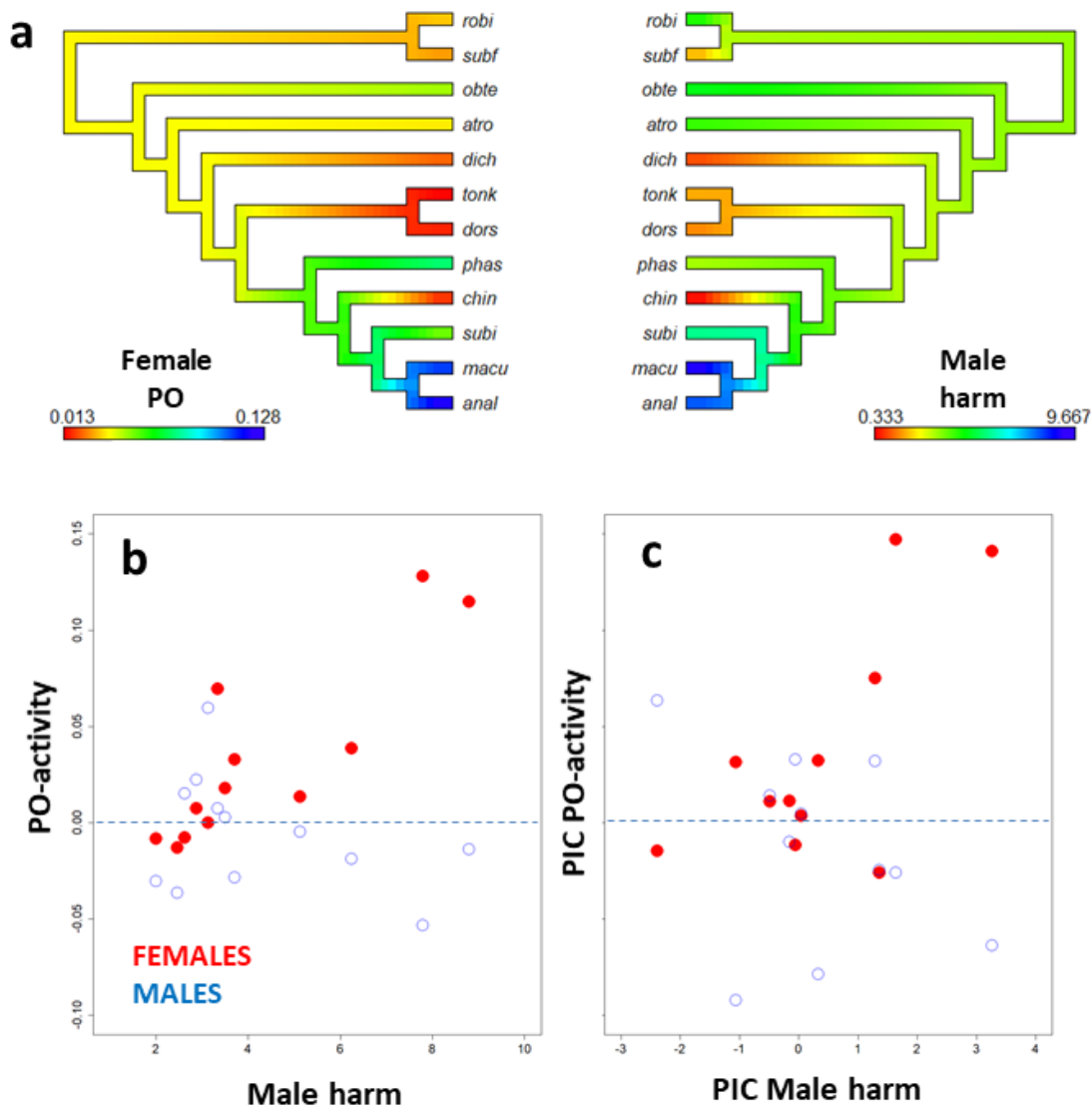
942 PO activity measured from whole-body samples of virgin (a) and mated (b) females from polygamous
 943 (green) monogamous (orange) and male-limited (blue) experimental evolution lines. The mating
 944 treatment significantly reduced female PO activity and male-limited and polygamous females had
 945 higher levels than monogamous females. Polygamous and monogamous females also differed
 946 significantly in the relationship between body mass and PO activity, suggesting that different allocation
 947 strategies evolved under the alternative mating regimes. Given are 95% confidence limit regression
 948 slopes. Males from the regimes did not express detectable levels of PO activity and showed no
 949 significant differences among regimes and mating treatments (Supplementary Table 1c). In the lower
 950 panels, sexual dimorphism in size-corrected PO activity is illustrated in each regime for (c) virgin and
 951 (d) reproducing beetles (Bayesian posterior modes and 95% credible intervals).



952

953 **Figure 4: Microevolutionary change in tolerance to bacterial infection during experimental**
 954 **evolution under alternative mating regimes.**

955 Response to bacterial infection was estimated by the change in mortality rate between individuals infected
 956 with two doses of bacteria and a sham control. When infected with the gram-positive bacteria *B. thuringiensis*,
 957 monogamous females (a, c) had significantly higher survival under infection compared with polygamous
 958 females (b, d), while virgin (a, b) and mated (c, d) females had similar responses. Shown are survival curves
 959 with 95% confidence limits based on all three replicate lines per regime and mating treatment. Virgin males
 960 (triangles) from monogamous (e) and polygamous (f) regimes did not show the strong differences seen in virgin
 961 females (circles), resulting in an apparent increase in sexual dimorphism in response to infection in the
 962 polygamy regime (compare panel e and f) (means \pm 1SE; lower dose = 1.0 OD, higher dose = 2.0 OD for females
 963 and 2.5 OD for males). When mated females were infected with the gram-negative bacteria, *P. entomophila*,
 964 which allowed assaying of in vivo bacterial counts in infected individuals, monogamous lines (g) again showed
 965 higher survival under infection compared with polygamous lines (h) (lower dose = 0.5 OD, higher dose = 1.0
 966 OD). (i) Counts of bacterial loads in females 12h post infection showed that difference in survival were likely
 967 not due to more efficient clearance of bacteria in monogamous lines. Bayesian posterior modes and 95%
 968 Bayesian credible intervals.



969

970 **Figure 5: Phylogenetic covariance between harmfulness of male genital morphology and PO**
 971 **activity in virgin male and female seed beetles.**

972 **(a)** Female PO activity and the harmfulness of male genitalia mapped on the phylogeny of the 12
 973 species used. Scores are given by color from blue (high harm/PO) to red (low harm/PO). Lower panels
 974 show correlations across species between male harmfulness and male (blue open) and female (red closed)
 975 PO activity, shown as **(b)** raw tip data and **(c)** phylogenetic independent contrasts (PICs).
 976 Species codes represent robi = *Amblycerus robiniae*; subf = *Zabrotes subfasciatus*; obte =
 977 *Acanthoscelides obtectus*; atro = *Bruchidius atrolineatus*; dich = *Bruchidius dichrostachydis*; tonk =
 978 *Megabruchidius tonkineus*; dors = *Megabruchidius dorsalis*; phas = *Callosobruchus phaseoli*; chin =
 979 *Callosobruchus chinensis*; subi = *Callosobruchus subinnotatus*; macu = *Callosobruchus maculatus*; anal
 980 = *Callosobruchus analis*.

981