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

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20

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24 **Abstract**

25 *Background:*

26 Sexual selection can have major effects on mating rates and sex-specific costs of mating and
27 may thereby influence sex-differences in immunity as well as associated host-pathogen
28 dynamics. Yet, experimental evidence linking the mating system to evolved sexual
29 dimorphism in immunity are scarce and the direct effects of mating rate on immunity are
30 not well established. Here, we use transcriptomic analyses, experimental evolution and
31 phylogenetic comparative methods to study the association between the mating system and
32 sexual dimorphism in immunity in seed beetles, where mating causes internal injuries in
33 females.

34

35 *Results:*

36 We demonstrate that female phenoloxidase (PO) activity, involved in wound healing and
37 defence against parasitic infections, is elevated relative to males. This difference is
38 accompanied by concomitant sex-differences in the expression of genes in the pro-
39 phenoloxidase activating cascade. We document substantial phenotypic plasticity in female
40 PO activity in response to mating and show that experimental evolution under enforced
41 monogamy (resulting in low remating rates and sexual conflict relative to natural polygamy)
42 rapidly decreases female (but not male) PO activity. Moreover, monogamous females have
43 evolved increased tolerance to bacterial infection unrelated to mating, implying that female
44 responses to costly mating may trade off with other aspects of immune defence, an
45 hypothesis which broadly accords with the documented sex differences in gene expression.
46 Finally, female (but not male) PO activity shows correlated evolution with the perceived
47 harmfulness of male genitalia across 12 species of seed beetles, suggesting that sexual

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48 conflict has a significant influence on sexual dimorphisms in immunity in this group of
49 insects.
50
51 *Conclusions:*
52 Our study provides insights into the links between sexual conflict and sexual dimorphism in
53 immunity at the molecular and phenotypic level and suggests that selection pressures
54 moulded by mating interactions can lead to a sex-specific mosaic of immune responses with
55 important implications for host-pathogen dynamics in sexually reproducing organisms.
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57 **Introduction**

58 Sex differences in immunity are widespread across animal taxa (1–4) and are believed to
59 reflect sex-specific selection and sexually dimorphic life histories (5–13). Sexual dimorphism
60 in immunity may have important consequences both for sex-specific rates of reproduction
61 and survival, with potential impact on population demography (14–18), and for the spread of
62 pathogens. For example, distinct male and female immune systems present more diverse
63 host targets (1,19,20) and this may influence both disease transmission, infection rates and
64 the expression and evolution of pathogen virulence (5,16–18,21–29).

65

66 Investment in immune defence is costly. These costs have most often been observed as
67 reductions in fecundity, effectively translating into reproduction-survival trade-offs in the
68 presence of pathogens (9,10,12,22,30–34). In polygamous species, where sexual selection on
69 males is intense, females are often predicted to gain more than males from investing in
70 survival and longevity at the cost of current reproduction and mating effort (3,9,35) and are
71 therefore also predicted to invest more in immunity than males (but see: (2,10,24,35–37)).
72 Sexual selection may also have pronounced direct effects on optimal investment in
73 immunity, as it may dictate the economics of reproduction (23,27,38,39) and lead to
74 elevated mating rates (40), which in turn may increase disease transmission (16,24,25,28).
75 Indeed, it has been suggested that sexual dimorphism in immunity should increase with sex-
76 differences in optimal mating rates and the strength of sexual selection
77 (5,13,21,23,27,41,42).

78

79 The effects of sexual selection on sex-differences in immune investment may be magnified in
80 systems where mating is harmful for females, through costs such as the transfer of

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81 pathogens during mating, transfer of immunosuppressive seminal fluid substances, or direct
82 physical injury (23,28,43–45). Such male-imposed mating costs are believed to be results of
83 sexual conflict driven by the different evolutionary interests of the sexes (6–8,46), in which
84 male adaptations evolve to increase reproductive success in competition with other males
85 despite impairing the health of their female mating partners. Females, in turn, evolve
86 counter-adaptations to alleviate the harm inflicted by males resulting in a coevolutionary
87 arms race between the sexes (23,43,46,47). Female immune responses may represent one
88 type of such counter-adaptation (23,27,48,49). This suggests that infections or harm on
89 females, induced by sexually selected male mating strategies, may be a significant selection
90 pressure on female immunity in polyandrous taxa (21,24,27,41,50). Hence, the evolution of
91 sexual dimorphism in immunity may in part be a result of male-imposed costs of mating in
92 females.
93
94 Yet, whether and how sexual conflict, or just mating *per se,* affect tissue-specific and general
95 immunity in the sexes is not well understood (5,22,23,44,51). It has, for example, been
96 suggested that tissue-specific (i.e. in the reproductive tract) immune responses upon mating
97 can lead to allocation trade-offs with systemic immunity (44,52), but few studies have
98 provided direct experimental evidence for a causal link between the mating system and the
99 evolution of sex-specific immunity trade-offs (2,37,49,50,53). To fill this empirical void, we
100 assessed how variation in the intensity of sexual conflict and mating rates in the seed beetle
101 *Callosobruchus maculatus* affects i) the evolution of male and female phenoloxidase (PO)
102 activity, a major component of invertebrate immunity involved in wound healing and
103 encapsulation of pathogens (54,55), and ii) associated immunopathological consequences of
104 bacterial infections unrelated to mating.

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105

106 Sexual selection is intense in *C. maculatus*, including both pre- and post-copulatory
107 processes (56–61), leading to sexual conflict over optimal mating rate and to male traits that
108 cause harm in females during mating (59,60,62,63). The male genitalia carry spines and
109 males with longer spines have greater fertilization success but the spines cause internal
110 injuries in females during mating, leaving females with melanized scars in the reproductive
111 tract as a result of the wound-healing process (59,60,62). Injurious copulations are wide-
112 spread in insects and may serve several functions, with the ultimate aim to increase male
113 competitive fertilization success (64,65). This may select for increased immune defence
114 locally in the female reproductive tract to enable efficient wound healing and limit female
115 susceptibility to sexually transmitted pathogens (66). Here, we show that PO activity in *C.*
116 *maculatus* females is high (see also: (48)) and responds dynamically to mating, while it is
117 very low in males. These sex differences are also mirrored in the expression of several key
118 genes regulating PO activity and related immune reactions. Experimental removal of sexual
119 selection and conflict led to rapid laboratory evolution of decreased female (but not male)
120 investment in PO activity. These changes were accompanied by the evolution of increased
121 female tolerance to bacterial infection unrelated to mating, suggesting a trade-off between
122 female responses to harmful mating and tolerance to other infections. The PO response was
123 paralleled at a macroevolutionary scale, signified by correlated evolution between male
124 genital morphology and sexual dimorphism in PO activity across 12 species of seed beetles.

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127 **Results**

128 ***Mating status and sex-biased gene expression in the prophenoloxidase-activating cascade***

129 The prophenoloxidase (proPO) activating cascade leads to the production of active PO,
130 which serves as an important defence in invertebrates against pathogenic bacteria, fungi and
131 viruses (54,55,67,68). Additionally, proPO has been implicated in cuticle tanning and other
132 developmental processes, as well as reproduction (reviewed in: (54,55,68)). PO aids in
133 wound healing and encapsulation of parasitic infections, and killing of pathogens by
134 generation of toxic secondary metabolites, such as reactive oxygen species (54,55,68–73).
135 However, the production of PO is strictly regulated (74,75) as it is both energetically costly
136 and the generation of toxic secondary metabolites can cause self-harm via
137 immunopathological responses (68,73,76,77), predicting that investment in PO activity could
138 incur costs to other fitness related traits (22,55,68,73). In Figure 1a we delineate the general
139 hypothesis for relationships between key components of the proPO cascade based on
140 functional annotations in insects and other invertebrates (reviewed in: (54,55,67,68,78)). To
141 gain insights into how sexual selection and conflict may affect investment in PO and other
142 correlated immunity traits in *C. maculatus*, we explored sex-biased gene expression of five
143 orthologs mapping to sequences of proteins functionally annotated for these key
144 components (Supplementary Table 1, Figure 1b). Spätzle processing enzyme (SPE) is involved
145 in the processes that cleaves proPO into active PO. We found that the expression of the *C.*
146 *maculatus* orthologs of both SPE and proPO are significantly female-biased in virgin adults.
147 Mating increased transcription of proPO in males leading to similar expression levels in the
148 sexes, whereas expression of SPE tends to increase in both sexes post mating and remains
149 female-biased (Figure 1b, Supplementary Table 1). These results suggest that females invest
150 heavily in PO activity via SPE and proPO. SPE also initiates the modification of spätzle (SPZ)

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151 and downstream TOLL-regulated antimicrobial peptides (AMPs), which offer inducible
152 immunity to pathogens. This may thus set the stage for a trade-off between PO
153 (encapsulation and wound healing) and SPZ (AMP-production) (see: e.g. (79,80) (Figure 1a).
154 Overactivation of the proPO cascade may also lead to the production of toxic secondary
155 metabolites (68,73), suggesting that excessive signalling via SPE to produce high levels of
156 both SPZ and PO may come at a cost to overall health (76,77). Interestingly, production of
157 serine protease inhibitors (serpins) via the TOLL-pathway exerts negative feedback and
158 control over the proPO cascade (81), and orthologs of both SPZ and the two putative serpins
159 that we identified in *C. maculatus* had strong male-biased expression (Figure 1b,
160 Supplementary Table 1). These patterns in gene expression thus suggest a putative
161 functional basis for sex-specific immunity via the pro-PO activating cascade, where we
162 hypothesize that females (relative to males) should invest more in PO activity in their
163 reproductive tract in response to harmful mating and the need for wound healing, but that
164 this investment might come at the potential cost of reduced AMP-production and/or toxic
165 side-effects of overactivation of the proPO cascade.
166 [FIGURE 1]
167
168 ***Sex-specific regulation of phenoloxidase activity***
169 We measured PO activity in homogenized whole-body samples of male and female larvae,
170 pupa and adults. The three life stages showed significant differences in mass-corrected PO
171 activity averaged across the sexes ($F_{2,33} = 17.7$, $p < 0.001$, Figure 2a). Some larvae showed
172 detectable levels of PO activity. Since we could not determine the sex of the larvae, sex-
173 differences in the larval stage can neither be confirmed nor rejected. Neither male nor
174 female pupae showed measurable levels of PO activity, whereupon there was a drastic and

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175 female-limited up-regulation in the virgin adults. Strikingly, virgin males did not show any
176 PO-activity, which was also the case for mated males (see further below), despite clear
177 expression of the proPO gene in males, especially following mating (Figure 1b). It seems that
178 proPO is not converted to PO in males to the same extent that it is in females, and other
179 proteins such as proPO activating factors (PPAFs), for which we could not confidently
180 identify gene transcripts, might be involved in regulating sex differences in how proPO is
181 converted into active PO. The observed effect size of sex on PO activity in virgin adults was,
182 Hedges' g = 2.08, which is high relative to what is typical in insects (mean Hedges' g = 0.55;
183 see (2)) and for animals in general (mean Hedges' g = 0.39; see (2)).

184

185 To further understand the function of the female-bias in adults, we explored how female PO
186 activity responds to mating. We mated females either only on day one of adult life
187 (treatment 100), on day one and two (110), on day one and three (101), or on all days (111)
188 and measured levels of PO activity subsequently on the third day (2h post mating in 101- and
189 111 females; ca. 24 and 48h post mating in 110- and 100 females, respectively). The
190 differences among the four treatments were substantial ($F_{3,52}$= 18.7, $p < 0.001$,
191 Supplementary Table 2). The PO activity was high in females when some time had elapsed
192 between mating and PO measurement (i.e. 100- and 110-females), while the levels were
193 near zero when PO activity was measured directly after mating (i.e. 101- and 111 females)
194 (Figure 2b). The treatment groups described above represent non-random samples of
195 females, as not all females can be made to remate on a given day. We therefore also
196 conducted a second experiment with random samples of 100 and 001 females. This showed
197 that the two treatments differed significantly (Mann-Whitney U test: W = 15, $p < 0.001$); 001
198 females had PO activity close to zero similar to 101 and 111 females in the first experiment,

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199 whereas 100 females had high PO activity similar to virgins, 100 and 110 females of the first
200 experiment (Figure 2). Hence, female PO activity decreases after mating but can be rapidly
201 recovered to initial levels post mating. These results accord with the observed female
202 upregulation of SPE in response to mating and the unconditionally high expression of proPO
203 in the female abdomen (Figure 1b, SI Table 1).
204 [FIGURE 2]
205
206 Using a subset of 25 females from the same population and same generation as the first
207 experiment, we performed a subsequent analysis of PO activity in oviposited eggs. This
208 analysis showed that decreases in female PO activity following mating is not due to PO
209 investment in offspring, as all five samples of pooled eggs showed very low (undetectable)
210 levels of PO activity, despite each sample representing about half of the lifetime egg
211 production of a single female. We found no evidence of a reproduction-immunity trade-off
212 as there was no relationship between the number of eggs laid by the females over the two
213 days of the first experiment and their subsequent measure of PO activity (Supplementary
214 Table 2). Although immunity-reproduction trade-offs are readily observed in insects
215 (9,12,13,22), PO investment does not always correlate negatively with fecundity (e.g.
216 (82,83)). Moreover, variation in overall phenotypic and genetic condition (84,85), as well as
217 the amount of male harm inflicted on females (86), could have masked a putative trade-off.
218 Alternatively, trade-offs with PO investment could materialize for other life-history traits
219 (9,30,87), and/or other components of immunity (22,55) (see: Figure 1a and further below).
220
221 ***Experimental evolution of phenoloxidase activity under different mating systems***

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222 To directly test the hypothesis that sexual selection and conflict over mating is causing the
223 observed sexual dimorphism in immunity in *C. maculatus*, we compared the levels of PO
224 activity in males and females from replicate experimental evolution lines maintained for 27
225 generations under one of three alternative mating regimes; natural **polygamy** (natural
226 selection and sexual selection – multiple mating); enforced **monogamy** (natural selection but
227 excluding sexual selection – single mating); and **male-limited selection** (applying sexual
228 selection but relaxing natural selection– multiple mating but female coevolution to reduce
229 male harm prevented). The lines are further described in the *Methods* section and in
230 (63,88,89). We predicted that females from polygamous lines that had evolved under
231 frequent multiple mating would invest more in PO activity than females from monogamous
232 lines, while the male-limited lines reveal the extent to which female PO activity may change
233 in the polygamous mating system via genetic correlation when selection acts mainly via
234 males. We also tested whether the direct effect of mating and reproduction on PO activity
235 had evolved under the different mating systems by for all lines comparing PO activity of
236 virgin and socially naïve individuals to that of beetles allowed to mate and reproduce for 48
237 hours in groups of 5 males and 5 females prior to the PO measurements.

238

239 We analysed the effects of experimental evolution regime crossed by mating treatment in
240 Bayesian mixed effect models using the MCMCglmm package (90) for R (91). Experimental
241 evolution line replicates, crossed with mating treatment, were included as random effects
242 (priors and model specification in Supplementary 3). The mating treatment decreased body
243 mass relative to the virgin treatment, revealing a sizeable investment in reproduction by
244 both sexes (SI Table 3a). While males did show an up-regulation of proPO gene expression in
245 response to mating (Figure 1b), they did not have any detectable levels of PO activity (n =

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246 354, SI Table 3c), confirming that PO investment is strongly female-biased in the adult stage
247 in *C. maculatus* (63). In females (N = 358 assays), the mating treatment significantly
248 decreased PO activity (ΔPO = -0.029 (-0.022; -0.037), $P_{MCMC} < 0.001$) but this effect was
249 similar in the three selection regimes (all pairwise interactions $P_{MCMC} > 0.6$) (Figure 3).
250 Importantly, evolution without sexual conflict under the monogamy regime had led to a
251 general decrease in female PO activity relative to the polygamy regime (ΔPO = -0.010 (-
252 0.002; -0.018), $P_{MCMC} = 0.030$), confirming a key prediction. The monogamy regime also
253 showed lower levels of PO activity compared to the male-limited regime, where females had
254 been kept under relaxed selection (ΔPO = -0.011 (-0.004; -0.020), $P_{MCMC} = 0.012$).
255 Accordingly, the polygamy and male-limited regime had similar levels of PO activity ($P_{MCMC} >$
256 0.8, Figure 3). Thus, as the expected number of matings decreased to a single mating in the
257 monogamy regime, the optimal female strategy was to decrease PO activity, in support of
258 the hypothesis that PO investment is costly and likely trades off against other female fitness
259 components (22,50,55,68,92). If immune defence is costly, a corollary from allocation theory
260 is that polygamous females should invest in PO in relation to their total energy reserves and
261 expected number of partners. In contrast, among monogamous females we expect the
262 evolution of decreased condition dependence due to their reduced need for PO activity. This
263 is also what we find; there was a positive relationship between female body mass and PO
264 activity in polygamous lines (slope = 0.011 (0.005; 0.016), $P_{MCMC} < 0.001$), whereas this
265 relationship was absent in monogamous lines ($P_{MCMC} = 0.48$), and this regime-difference in
266 the condition dependence of PO investment was significant (Δslope = 0.007 (0.001; 0.013),
267 $P_{MCMC} = 0.026$, Figure 3).
268 [FIGURE 3]
269

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270 Again, however, a fecundity cost of high PO activity was not apparent when comparing
271 regimes; offspring production in the reproducing treatment was higher for females from the
272 polygamy regime (showing higher levels of PO activity) than for monogamous females (with
273 lower levels of PO activity) (SI Table 3b).
274

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

276 To explore other possible immunological consequences of mating system and sexual conflict,
277 which could be driven by trade-offs between investment in PO and other components of
278 immunity (Figure 1), we measured survival in the monogamy and polygamy lines when
279 exposed to bacterial infection in abdominal tissue adjacent to the reproductive tract.
280 Females (total n = 1060, 24-48h past adult eclosion) were either virgin or mated prior to
281 being infected with one of two doses (OD1 or OD2) of the entomopathogenic gram-positive
282 bacteria, *Bacillus thuringiensis,* or a sham control (pricking with a sterilized needle dipped in
283 PBS buffer). We analysed survival in mixed effects Cox proportional hazard models using the
284 coxme package (93) for R, with regime and mating treatment as fixed effects and replicate
285 lines as random effects. We also confirmed results by using the MCMCglmm package (90) to
286 apply Bayesian mixed effect models on a binomial response variable (dead/alive on day 5
287 post infection), which allowed us to add fully crossed random effects (line by treatment) in
288 the analysis (Full statistical summaries in Supplementary Tables 4a, b).
289

290 Females from the polygamy regime showed lower survival under bacterial infection
291 compared to females from the monogamous regime (X_2^2 = 13.7, P = 0.001, Figure 4a-d). This
292 result, albeit correlative, is in line with the hypothesis that the evolution of female immunity
293 responses to expected harmful mating may trade-off against general susceptibility to

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294 infection. Mating by itself led to an increase in mortality (X_1^2 = 63.6, P < 0.001). However,
295 there was no significant effect on susceptibility to infection of either mating status (X_2^2 = 1.2,
296 P = 0.56) or the interaction between evolution regime and mating status (X_2^2 = 0.14, P =
297 0.93, Figure 4a-d). Although somewhat surprising, this result is not inconsistent with a trade-
298 off between female PO investment in the reproductive tract and vulnerability to systemic
299 infection caused by other pathogens, as also virgin females display high PO activity and high
300 expression of genes in the proPO cascade prior to being mated (Figures 1b & 2). Virgin males
301 from monogamous and polygamous regimes (which do not seem to invest in PO at all) did
302 not show any strong differences in their response to bacterial infection (assessed in a
303 separate experiment, X_2^2 = 0.94, P = 0.63, Figure 4e,f). However, although we analyzed the
304 same number of evolution lines in the male experiment, the total number of individuals
305 analyzed was smaller (n = 270 for virgin males compared to n = 493 for virgin females),
306 limiting direct comparisons between the male and female assays. Nevertheless, the male
307 experiment did reveal an overall effect of the bacterial injection (X_2^2 = 7.77, P = 0.021) and
308 significantly greater survival of polygamous males (X_2^2 = 6.63, P = 0.010) (SI Table 4c).
309

310 To gauge the generality of these results, and to further investigate whether the higher
311 survival of monogamous females under bacterial infection was due to more efficient
312 clearing of the bacterial infection (greater resistance), or because they were better at
313 withstanding it (greater tolerance) (94), we infected once-mated polygamous and
314 monogamous females with the gram-negative bacteria *Pseudomonas entomophila* using the
315 same protocol as described above. The *P. entomophila* strain used is resistant to the
316 antibiotic ampicillin. This allowed us to screen a subset of females collected 12h post start of
317 infection exclusively for *P. entomophila* by culturing female cell tissue on Luria agar plates

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318 with ampicillin. Again, females from the polygamy regime showed higher susceptibility to
319 bacterial infection (X_2^2 = 16.6, P < 0.001, total n = 288, Figure 4g,h, SI Tables 4d,e). However,
320 there was no significant difference in bacterial load among the evolution regimes (P_{MCMC} >
321 0.2, n samples = 63, n females = 189, Figure 4i, SI Table 4f), suggesting no large differences in
322 the ability of females to clear the bacterial infection. This last result does not support an
323 allocation trade-off between the production of PO and AMPs, and may be more consistent
324 with increased mortality due to toxic secondary metabolites resulting from overexpression
325 of the proPO activating cascade by polygamous females (68,75–77) (Figure 1a). However,
326 more work is needed to pin-point the exact mechanism underlying the differential mortality
327 among monogamous and polygamous females. More generally, our results are consistent
328 with the hypothesis that sexual conflict and harmful mating can lead to increased
329 vulnerability to infection in females as a result of sex-specific trade-offs between different
330 components of immunity (23,44,52).

331 [FIGURE 4]

332

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

334 We explored whether macroevolutionary transitions in sexual dimorphism in immunity could
335 be driven by the evolution of mating interactions and the harmful morphology of male
336 genitalia in this group of insects (23). We measured PO activity in virgin males and females of
337 12 species of seed beetles. There was pronounced sexual dimorphism and female-limited
338 expression in many species (SI Figure 5a). To quantify harmfulness of the male genitalia in
339 each species, we asked two expert and ten naïve biologists to rate pictures of male genitalia
340 for the perceived harm they cause in the female reproductive tract (SI Figure 5b).

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341 Importantly, earlier work has shown that male harm assayed in this manner correlates
342 positively with the amount of scarring that occurs in the female copulatory tract after mating
343 (23). Species differences explained 61% of the total variation in rater scores and scores were
344 highly correlated between experienced and naïve raters ($r = 0.83$), suggesting that raters
345 generally agreed on the classification of male harm. Female and male PO activity, as well as
346 male harm, showed moderate phylogenetic signals (Blomberg's $K = 0.68$, 0.52 and 0.54,
347 respectively (95)). Hence, we applied a phylogenetic generalized least squares regression
348 (PGLS) based on species means using the ape package (96) for R, accounting for phylogenetic
349 dependencies using Ohrstein-Uhlenbeck estimation and an extant seed beetle phylogeny
350 (97,98). There was significant positive covariance between male harm and female PO activity
351 ($\alpha = 6.70$, standardized slope = 0.83, $df_{12,10}$, $P < 0.001$, SI Table 5a). Moreover, the covariance
352 between male harm and male PO activity was not significant and opposite in sign ($\alpha = 2.92$,
353 standardized slope = -0.57, $df_{12,10}$, $P = 0.08$, SI Table 5b). These analyses, together with our
354 experimental findings, implicate sexual conflict as a driver of macro-evolutionary divergence
355 in sexual dimorphism in immunity (Figure 5).
356 [FIGURE 5]
357

358 Discussion

359 Sexual selection can result in increased male harm to females during mating (22,29,32),
360 either through direct injury or infection with pathogens, and this should in theory favour
361 increased female investment in immunity when female lifetime reproductive success is
362 elevated by increased longevity (5,22–24,27,35,39). Here, we provide a suite of experimental
363 and comparative data collectively showing that sex-differences in immunity can be
364 modulated by sexual conflict in a species where costs of mating are conspicuous. This

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365 conclusion is based upon observations of (1) sex-biased expression of genes in the proPO
366 activating cascade (Figure 1), (2) a female-bias in PO activity which is substantially higher
367 than what is typical in insects, (3) female-limited phenotypic plasticity in PO activity in
368 response to mating (Figure 2), (4) female-limited microevolutionary changes in immunity
369 traits in response to experimental manipulation of the mating system and hence sexual
370 conflict (Figures 3 & 4), and (5) correlated evolution between male genital morphology and
371 female PO activity across species (Figure 5).

372

373 While previous studies have quantified female immune responses post mating
374 (5,5,22,23,45,50,53,99,100), it often remains unclear whether male harm via genitalia or
375 ejaculatory compounds (i.e. sexual antagonism) drive such responses, or whether they
376 represent independent female optimization of the trade-off between current and future
377 reproduction (5,22,23,27,49,101). Here, we directly manipulated the level of sexual selection
378 and conflict, which is relatively well understood in *C. maculatus* (e.g. (47,48,59,62,102–105)),
379 and found a clear female-limited PO response, while no correlation between female
380 reproductive investment and PO activity was detected. Hence, our data point to male harm
381 inflicted during mating as the driver of female PO investment. In this system, the inflicted
382 harm by a male on his female mating partner is positively correlated to his success in sperm
383 competition (29), presumably because seminal fluid substances (66) that benefit males in
384 sperm competition (62) pass more rapidly into the female body if the copulatory duct is
385 ruptured (32). However, these wounds may leave females at a risk of systemic infection with
386 pathogens (36), suggesting a need for healing these injuries via a PO-mediated, potentially
387 costly (68,73,76,77), reaction.

388

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389 We hypothesized that these effects could have consequences for female susceptibility to
390 infections unrelated to mating via trade-offs between PO activity and other components of
391 immunity in the prophenolixidase activating cascade, such as the production of AMPs (Figure
392 1). This prediction was offered correlative support by the observation of increased
393 susceptibility to bacterial infection in females from the polygamous mating regime (Figure
394 4). However, our results do not allow us to confidently distinguish between several, mutually
395 inclusive, hypotheses regarding the exact mechanistic basis for the increased mortality of
396 polygamous females. We did not find a difference in bacterial load between polygamous and
397 monogamous females infected with the gram-negative bacteria *P. entomophila*, suggesting
398 no large differences in the ability to clear infection and therefore also no large differences in
399 the production of AMPs used to fight bacterial infection; a result that does not support a
400 trade-off between the production of AMPs and PO in polygamous females. The result from
401 this experiment is hardly conclusive, however. Another possibility is that the need for high
402 PO activity in the reproductive tract of polygamous females led to a harmful "overactivation"
403 of the proPO activating cascade upon bacterial infection and the simultaneous need for AMP
404 production (e.g. (106)). Indeed, while such overactivation could mask allocation trade-offs by
405 attending the dual need of producing PO and AMPs, it may have caused an inflammatory
406 response with increased mortality of polygamous females as a result. The proPO activating
407 cascade can have detrimental immunopathological consequences via the production of toxic
408 secondary metabolites and needs to be strictly regulated (76), and severe bacterial infection
409 can kill the organism also via side-effects of excessive melanization (68,73). Future
410 experiments are needed to pin-point the exact mechanistic basis underlying our results,
411 preferably including detailed measures of tissue-specific immunity responses as we here
412 measured whole-body samples.

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413

414 Interestingly, polygamous females suffered increased mortality when infected with gram-
415 positive and gram-negative bacteria, with only the former considered important in activating
416 immune responses via TOLL, while the latter is thought to elicit immune responses mainly
417 via the Imd pathway (78), which does not have a clear connection to the proPO activating
418 cascade. This result might thus speak against immunity trade-offs between components in
419 the proPO activation cascade as a general mechanism explaining the observed differential
420 mortality between polygamous and monogamous females. However, several studies on
421 invertebrates have now demonstrated cross-talk between the TOLL and Imd pathway
422 ((55,78,107–109)), and that the proPO cascade can be readily activated by gram-negative
423 bacteria (74,109,110). Indeed, TOLL has even been directly implicated in regulating sexual
424 dimorphism in immunity to gram-negative bacteria in fruit flies (111), suggesting that the
425 consistent difference in mortality of monogamous and polygamous females infected with
426 the gram-positive *B. turingiensis* and gram-negative *P. entomophila* may yet be rooted in
427 differential usage of the proPO activating cascade.

428

429 Male reproductive success in polyandrous mating systems is typically maximized by a shift
430 towards current reproduction in the female mating partner, as this would increase the
431 likelihood of the male siring a larger fraction of the offspring produced by the female
432 (5,16,23,24,44). These ideas predict that males should evolve to manipulate females to
433 invest in current reproduction at the expense of reduced immunity and longevity (22,23). In
434 line with these predictions, males with longer genital spines, that inflict more harm during
435 mating, sire more offspring in *C. maculatus* (59,62) and seem to stimulate female fecundity
436 (*unpublished data*). Moreover, the male ejaculate regulates female immunity post mating in

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437 *Drosophila*, guppies, mice and humans (44,45,51,52,112,113), although it often remains
438 unclear to what extent the effects are detrimental or beneficial to the female overall
439 (5,22,23,27,114). It has even been suggested that males may gain fitness benefits by
440 transferring sexually transmitted diseases that trigger shifts in female allocation towards
441 current reproduction (21,115), but this possibility lacks empirical support (116). In other
442 insects, female PO either increases or decreases post mating and it has been suggested that
443 in species where mating downregulates female PO activity, males corrupt the female
444 immune function (23). While our results do not refute this hypothesis, they are also
445 consistent with *C. maculatus* females being "primed" for harmful mating and that PO activity
446 in females initially decreases post mating as a result of wound healing but is then quickly
447 restored. Such female anticipatory immunity activation has been observed in *Drosophila*
448 (117,118) and bed bugs (119).
449

450 *Conclusions*

451 When mating rate affects both sexual dimorphism in immunity and infection rates, this can
452 result in intricate eco-evolutionary dynamics with demographic consequences for both host
453 and pathogen (5,16,21–25,27). Our study suggests that sexual conflict over mating rate can
454 drive sexual dimorphism in immunity and that allocation to different components of
455 immunity may play an important role in mediating effects of mating on females. In
456 *Drosophila*, mating increases immune responses in reproductive tissue, and in most insects
457 mating decreases general immunity, but causality typically remains unclear (22,23). Our
458 results imply that baseline PO activity decreases in *C. maculatus* females as a genetic
459 response to the alleviation of sexual conflict and harmful mating. Moreover, monogamous
460 females, that evolved a reduced investment in PO activity relative to naturally polygamous

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461 females, showed an associated evolutionary increase in tolerance to bacterial infection in
462 abdominal tissue adjacent to the reproductive tract, effects not seen in their conspecific
463 males. This suggests that sex-specific trade-offs determine the mosaic of immune
464 investment and that sexual selection and conflict affect the economics of these trade-offs.
465 This complexity may explain some of the discrepancies found in the literature concerning
466 female immune responses to mating (reviewed in: (5,23,27)) and motivates further
467 explorations of the selection pressures affecting sexual dimorphism in immunity.

468

469 ## Methods

470 ### ***Study populations***

471 *Callosobruchus maculatus* females lay eggs on seeds and larvae burrow into the seed where
472 the entire development occurs. Beetles emerging from seeds are reproductively mature and
473 require neither water nor food to reproduce successfully (e.g. (120,121). Adults typically die
474 7-14 days after emergence in the absence of food or water (e.g.(122). All experiments used
475 beetles originating from a genetic stock that was originally sampled in Lome, Togo, in 2010,
476 and subsequently maintained as 41 isofemale lines in the laboratory to maintain the genetic
477 variation present in the original population (123), before being mixed into a large, outbred,
478 and genetically diverse experimental population (N ~500). This genetic stock has been used
479 in quantitative genetic designs (e.g. (102,123–125), artificial selection experiments (126),
480 and experimental evolution (63,88,89) to demonstrate substantial sex-specific standing
481 genetic variation in behavior, morphology, life-history and life time reproductive success, as
482 expected given that the lines originate from the center of the species range (127).

483

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484 The experimental evolution lines used to study the effect of the mating system on the
485 evolution of sexual dimorphism in immunity are thoroughly described in (63,88). In brief, the
486 lines were maintained under standard temperature (29°C), humidity (50%RH) and light cycle
487 (12L: 12D), and were reared on the preferred host plant (127) *Vigna unguiculata* (black-eyed
488 bean). There are three replicate "Monogamy" lines, three "Polygamy" lines and two
489 replicate "Male-limited" lines. Effective population size for the lines in each regime was kept
490 approximately equal ($N_e \approx 150$; $N_{Male\text{-}limited} = 200$, $N_{Monogamy} = 246$, $N_{Polygamy} = 300$) and the
491 number of beans provided as egg laying substrate in each regime was standardized to give
492 the same, relatively low, juvenile density (2-4 eggs/bean) to minimize (and equalize) larval
493 competition (63). To implement the different regimes, selection was only applied for the first
494 two days of adult life. However, the reproductive output over these first days typically
495 corresponds to half of the total lifetime reproductive output (D. Berger, unpublished data).
496 The regimes show differences consistent with generally positive effects of sexual selection
497 on genetic quality in terms of increased female reproductive success and population
498 productivity in polygamy lines relative to monogamy lines at generations 16 and 20,
499 respectively (63). They also show differences in sexually selected male pre- and post-
500 copulatory traits (88,89).
501

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

503 To assay the effects of sex and mating status on the expression of relevant genes, we used
504 data previously published in (128). Briefly, RNA sequencing (Illumina TruSeq) was used to
505 test for sex differences in gene expression in virgin and mated age-matched beetles,
506 separately for reproductive and non-reproductive tissues (i.e. abdomen and head & thorax,
507 respectively). In the mating treatment, RNA was extracted 24h after mating. We pooled six

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508 individuals of each sex, tissue and treatment and replicated these pools three times. The
509 transcriptome was assembled *de novo* (129), and differential expression analysed using
510 edgeR, as described in(128). The candidate PO genes were detected using BLAST (tblastn
511 search in the TSA database for *C. maculatus*, using the protein sequences as query) and here
512 we report the ones with a significant sex difference in expression (with a false discovery rate
513 adjusted p-value < 5%) in the virgin beetles in either tissue category.
514

515 ***Phenoloxidase assays***

516 Individual beetles were homogenized by 20 seconds of grinding with a pestle in an
517 Eppendorf tube containing 20 µl Phosphate Buffered Saline (PBS). Samples were kept on ice
518 until centrifuged at 17g for 10 min at 0°C, and the supernatants (10 µl) were stored at -80°C
519 prior to the assay of PO activity. The frozen homogenates were analysed by an investigator
520 uninformed of the samples' identity and treatment affiliation, i.e as blind tests. Due to the
521 small volume of each sample and high background due to the crude protein extract, the
522 assay was first developed and optimized to ensure that proper enzyme kinetics were at
523 hand, and phenylthiourea could completely block the activity (see Supplementary
524 Information 6). In preliminary experiments the beetle homogenate was preincubated with
525 curdlan (a β-1,3-glucan), trypsin or chymotrypsin to fully convert all zymogenic proPO to the
526 active enzyme PO before assay of enzyme activity. However, the frozen homogenates did
527 not show any increased PO activity after activation, indicating that the preparation method
528 such as freezing at -80˚C had converted all proPO into active enzyme PO (Supplementary
529 Information 6). Dopamine, L-Dopa and 4-methylcathecol+hydroxyproline ethyl ester were
530 each tested as substrate for *Callosobruchus* PO, and dopamine was shown to be the most
531 efficient substrate and was used in the further experiments (rough estimates of Km in this

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532 crude homogenate for L-dopa Km≈ 6.3 mM, and for dopamine Km ≈ 0.2 mM, while 4-
533 methylcathecol + hydroxyproline ethyl ester as substrate did not show linearity). For the
534 experimental samples, six samples of beetle homogenate at a time were randomly chosen
535 and thawed. After thawing, each individual beetle homogenate (3 µl) was incubated
536 together with 7 µl PBS and 50 µl dopamine [10 mM in H_2O] at 22°C. The reaction proceeded
537 for 15 minutes after which 60 µl H_2O was added to terminate the reaction and after
538 centrifugation at 16000 x g for 1 min the absorbance at 420 nm was recorded. The enzyme
539 assay was first developed to ascertain zero order kinetics, and due to the crude source of
540 enzyme individual blank controls (without substrate) had to be measured before and after
541 the reaction for each sample. This blank control was assayed containing 3 µl beetle
542 homogenate, 7 µl PBS and 50 µl H_2O, and was incubated and measured as the samples
543 above. The enzyme activity is expressed as increase in absorbance at 420 nm per minute in
544 the focal sample relative to its blank control (∆A420/min).
545
546 ***Sex-specific ontogenetic regulation of phenoloxidase activity***
547 The eggs laid by the females in the mating status experiment (below) were followed through
548 ontogeny. We sampled a total of 20 final instar larvae, 20 pupae and 14 adults. Larvae of *C.*
549 *maculatus* could not be sexed. Pupae were sexed by abdominal morphology, for a total of 10
550 male and 10 female pupae. Virgin adults were collected as virgins within 0-36 hours post
551 emergence. All individuals were weighed and measured for PO activity. We analysed
552 differences between developmental stages by adding mass of the tissue analysed as a
553 covariate in an ANCOVA. As we could not determine the sex of larvae, we performed one
554 model that averaged effects across the sexes and one model where we excluded larvae and
555 could retain sex. Both models showed significant differences between life stages.

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556

557 ***Female phenoloxidase activity in response to mating.***

558 We used males and females from the Lome base population, reared at standard conditions.
559 All adults were virgin and between 24-48 hours old at the start of the experiment. On day
560 one, 120 females were individually placed in small 30mm diameter petri dishes together
561 with two males, in three separate bouts (40 females at a time). Matings were observed and
562 mated females were immediately removed and placed into a 90mm diameter petri dish
563 containing black eyed beans allowing females to oviposit. In total, 114 of the 120 females
564 mated successfully over an observation period of 20 minutes per bout. A random set of 35 of
565 these females were assigned to treatment 100 (mating on day one and then reproduction in
566 isolation until being measured for PO activity on day three). The rest of the females were
567 given the opportunity to mate on day two and day three, but all females did not mate on all
568 days. This resulted in four treatment groups; 100, (mated on day 1 only), 110 (mated on day
569 1 & 2), 101 (mated on day 1 & 3) and 111 (mated on all days). Approximately two hours after
570 the final mating on day three, all females were weighed and then measured for PO activity
571 as described above. Measuring PO activity is time-consuming, and since preliminary analyses
572 of the first batch of females suggested sufficient power to detect effects of mating status
573 (see Figure 2), all females were not measured. The following sample sizes were attained for
574 each treatment; 100: 15, 110: 7, 101: 13, and 111: 23 females. The treatment groups
575 described above from the first experiment represent non-random samples of females, as not
576 all females can be made to remate on a given day. We therefore conducted a second
577 experiment with random samples of 100 (n = 15) and 001 (n = 15) females to confirm the
578 main result from the first experiment. We counted the number of adult offspring produced
579 by each female over the 48h of egg laying in the first experiment. We analysed the effect of

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580 mating status and number of offspring produced, including their interaction, on female PO
581 activity in an ANCOVA. Female body mass at the time of homogenization was included as a
582 covariate.
583
584 To determine whether female PO is allocated to eggs, 10 matured eggs per female were
585 dissected out from 25 virgin females for a total of five samples containing 50 eggs each
586 (corresponding to approximately 50% of the lifetime production of eggs of a single female).
587 Samples were weighed and then subjected to the same crushing and centrifuging protocol as
588 the mated females before being frozen at -80 °C and later measured for PO activity.
589
590 ***Experimental evolution of phenoloxidase activity under alternative mating regimes***
591 The experiment was performed following 27 generations of experimental evolution and one
592 subsequent generation of common garden (polygamy) selection through standard culturing
593 to remove any potential influence of parental environmental effects. PO activity was
594 measured in the whole body of single male and female beetles from two replicate lines from
595 each mating regime (6 lines in total). To manipulate the reproductive status of the beetles,
596 newly emerged virgin adults (0-48h old) were either placed together in 90mm diameter
597 petri-dishes in groups of five males and five females that were allowed to reproduce
598 ("Reproducing" treatment), or in petri dishes with 5 males and 5 females but individually
599 isolated in aerated Eppendorf tubes ("Virgin" treatment). All petri dishes contained black
600 eyed beans, so that all beetles experienced the olfactory stimuli of the host beans, but only
601 reproducing females could oviposit on the beans. After 46h, individuals were weighed
602 before being put through the protocol to measure PO activity (see above). Beans from the
603 mating treatment were stored until adult offspring emerged. Offspring were frozen and

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604 20˚C and later counted to estimate allocation to reproduction in all regimes. We set up the
605 experiment in two separate batches one week apart in time, with each batch containing one
606 replicate line of each evolution regime. We analysed differences among evolution regimes
607 and mating treatments in Bayesian mixed effect models implementing Markov chain Monte
608 Carlo simulations using the MCMCglmm package (90) in R (91). We ran separate models for
609 males and females as PO activity was virtually undetectable in males. Evolution regime and
610 mating treatment, including their interaction, were added as fixed effects and body mass
611 was added as a covariate to control for the amount of tissue analysed as we used whole-
612 body samples. We first tested for presence of a higher order interaction between mating
613 status and evolution regime, which was non-significant and removed. We then evaluated
614 significance of main effects by comparing the posterior distributions of marginal means for
615 two groups in a given comparison (e.g. comparing mean PO activity of monogamous and
616 polygamous females, averaged over the two mating statuses). In follow-up analyses we also
617 assessed interactions between female body mass and mating status and evolution regime
618 (to test for condition-dependence of PO activity; see Results). We blocked out effects of
619 batch by adding it as a fixed effect. Similarly, we also blocked out the potential effect of
620 freezing some individuals before homogenizing samples, something that had to be done for
621 logistic reasons. Replicate line crossed with mating treatment, and adult mass when
622 appropriate, were always included as random effects when estimating effects of evolution
623 regime on PO activity. We used weak and unbiased priors for the random effects and ran
624 models for 3,000,000 iterations, preceded by 100,000 burn-in iterations that were discarded,
625 and stored every 3,000th iteration (thinning), resulting in 1,000 uncorrelated posterior
626 estimates of the fixed effects upon which we calculated Bayesian P-values and 95% credible

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627 intervals. Prior specification and MCMC settings were the same for all models (exemplified in
628 Supplementary Table 3c).
629

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

631 At generation 50, we collected beetles from each of the three replicate populations of the
632 Monogamy and Polygamy regime and then maintained them under common garden
633 conditions (natural polygamy) for one generation to minimize environmental parental
634 effects. To measure evolved vulnerability to a bacterial pathogen, we first isolated 2-day-old
635 experimental virgin females from each of the lines and paired them individually with a single
636 male from their own line for 5 hours. Simultaneously, we also collected another subset of
637 females that were held as virgin throughout the experiment. On day three post eclosion, we
638 infected females with a strain (DSM 2046) of the entomopathogenic gram-positive bacteria
639 *Bacillus thuringiensis,* described in (130). Beetles were first anesthetized with carbon-dioxide
640 and then pricked at the lateral side of the lower abdomen, using a 0.1mm minutien pin (Fine
641 Science Tools) dipped in overnight bacterial suspension of 1 OD or 2 OD (subcultured from
642 an overnight culture of the bacteria). We performed sham infection with a pin dipped in
643 sterile PBS solution. Following the start of the infection (or sham infection), we isolated
644 females individually in 24 well-plates. We monitored individual survival at every 12 hours
645 until 48 hours post infection and daily around 6pm for the next 8 days. Females still alive 10
646 days post infection (less than 30%) were right-censored in the subsequent survival-analysis.
647 In a separate experiment, we also measured survival of infected 3-day old virgin males as
648 described above.

649 At generation 54, we again collected mated females from two randomly selected replicate
650 populations each of Polygamy and Monogamy and maintained them under common garden

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651 conditions. In the subsequent generation (Gen 55) we collected virgin females from each
652 regime. We first mated two-day old females with a male from their own population. We
653 then infected the females with a 0.5OD (52.5 ± 19.3 cells/beetle) or 1.0OD (237.5 ± 124.7
654 cells/beetle) solution of the gram-negative bacteria *Pseudomonas entomophila* using the
655 same protocol as described above for *B. thuringiensis* (note that we could not calculate exact
656 cell counts for *B thuringiensis* as the strain used lacked an antibiotic marker). Following the
657 start of infection, we housed females individually in the 24 welled plates. Survival was first
658 observed after 12 hours and a subset of beetles were taken out for bacterial load assay
659 described below. We measured survival up to 120 hours after the start of infection.

660 The *P. entomophila* strain used is resistant to the antibiotic ampicillin. This allowed us to
661 screen the females collected 12h post infection exclusively for *P. entomophila* by plating
662 their whole-body extract homogenized in sterile PBS buffer on LB agar plates with ampicillin
663 (0.1mg/ml), and subsequently counting bacterial cultures on the plates to estimate bacterial
664 load. We first collected 3 surviving females 12hours after start of infection and transferred
665 them to a micro-centrifuge tube. We then washed the three beetles together with 70%
666 ethanol twice. Following the ethanol wash we again washed them with sterile water once.
667 Subsequently, we added 90 µl of PBS and crushed the beetles together using a sterile micro-
668 pestle. From this master-stock solution we made dilutions up to 10^{-5} in 96-welled plates. We
669 spotted 3ul of each dilution on Luria agar plates with ampicillin. We kept the plates over
670 night at 27°C and counted distinguishable *Pseudomonas entomophila* colonies. From the
671 number of colonies, we calculated the bacterial load per female beetle and used that for
672 further analyses. In total we calculated load for 8 samples per line and bacterial
673 concentration. One sample was lost, resulting in a total of 63 samples (each based on 3
674 females). Analyses described in the Results and model specifications in Supplementary 4.

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675

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

677 We measured the PO activity of 5 virgin males and 5 virgin females of each of the 12 species
678 (see Figure 5) using whole-body samples. All individuals were less than 48h old post adult
679 emergence. As the species differ widely in body size, we modified the amount of PBS buffer
680 added at homogenization to retain more equal concentration of tissue for all species in the
681 original samples to be analysed for PO activity.

682

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

698

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699 We analysed the covariance between harmfulness of the male genitalia and male and
700 female PO activity based on species means across the phylogeny using phylogenetic least
701 squares (PGLS) regression with Ohrstein-Uhlenbeck correction implemented in the ape
702 package(96) for R (model specification and output in Supplementary Table 4). All variables
703 were variance standardized in the analyses. Given the uncertainty of exact branch lengths,
704 we set all branches to unit length. PO measurements were divided by the concentration of
705 tissue in each sample prior to analysis.
706

707 ## *Declarations*

708 ### *Author Contributions*

709 IS performed all PO activity assays. EP performed experiments on mating status and
710 ontogeny. JLR and EP collected data for species comparisons. JB and IMA maintained the
711 selection lines. QC performed the experiments on PO activity in the lines. BB, DBa and IK
712 planned and performed measures of responses to bacterial infection in the evolution lines.
713 EI and AS performed the bioinformatic analyses. DBe analyzed all other data together with
714 JB. QC, JB, EI and DBe produced the figures. DBe planned and conceived the study with
715 considerable input from IS, GA and IK. DB wrote the first draft of the manuscript with input
716 from all authors.

717 ### *Competing Interests Statement*

718 The authors declare no competing interests

719 ### *Ethics Statement*

720 This research was conducted according to national legislation. No permits are needed for
721 research on invertebrates.

722 ### *Data accessibility:*

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723 All data will be uploaded to the Dryad data repository upon final acceptance.

724 ***Consent to publish***

725 All authors and institutions have approved the submission

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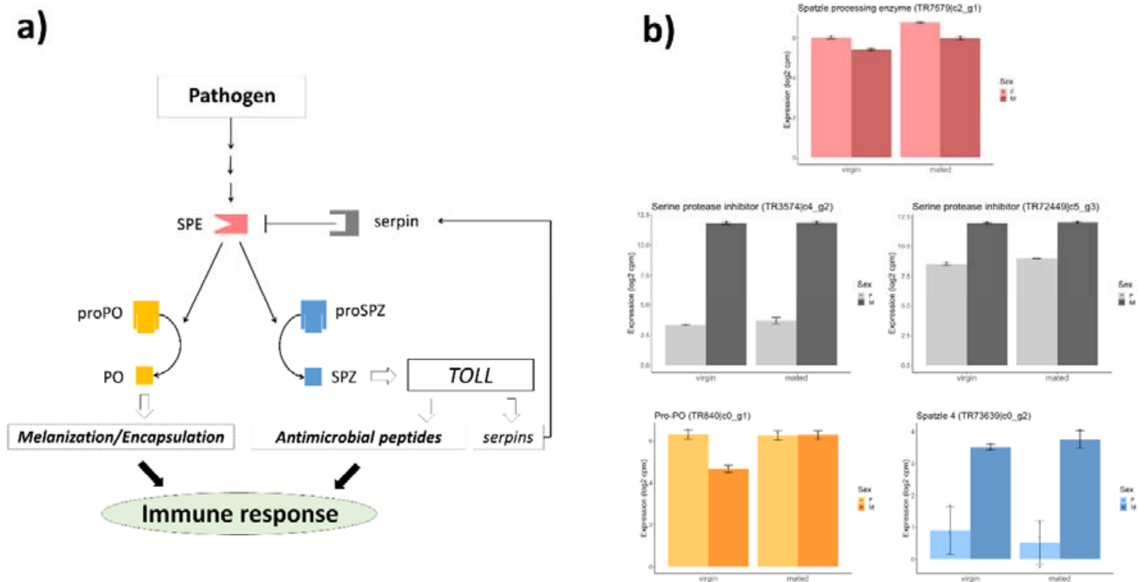

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1045 **Figure 1: Sex-biased gene expression in the proPO signalling cascade.**
1046 In **a)** schematic representation of key proteins in the proPO activating cascade, based on previous
1047 studies of insects and other invertebrates (reviewed in: (54,55,67,73,78)). In **b)** sex-bias and effects of
1048 mating status on gene expression in the abdomen for *C. maculatus* orthologs from published data
1049 (128) mapped to the sequences of the functionally annotated proteins. Full results in Supplementary
1050 Table 1. Spätzle processing enzyme (SPE: pink) initiates cleavage of proPO (yellow) into active PO,
1051 which ultimately leads to wound healing as well as encapsulation and killing of foreign pathogens.
1052 However, SPE also regulates the production of Spätzle protein (SPZ) from proSPZ (blue), which
1053 ultimately leads to increased production of antimicrobial peptides (AMPs) via the TOLL pathway,
1054 which offers inducible immunity against pathogens, thus setting the stage for an allocation trade-off
1055 between PO-activity and AMP-production. Overactivation of the proPO cascade has toxic side-effects
1056 via the production of secondary metabolites, suggesting that overproduction of SPE may come at a
1057 cost to overall health. Here, production of serine protease inhibitors (serpins: grey) in the TOLL-
1058 pathway exerts negative feedback and control over the cascade. **b)** *C. maculatus* females show
1059 higher expression of SPE (pink) and proPO (yellow) as virgins. Males show higher expression of
1060 proSPZ (blue) and serpins (grey). These patterns in gene expression suggest a mechanistic basis for
1061 sex-specific immunity trade-offs between different components in the pro-PO activating cascade,
1062 where females are predicted to invest more in PO-activity (wound healing and potentially
1063 encapsulation of pathogens transferred at mating) in their reproductive tract in response to mating,
1064 at the potential cost of reduced inducible immunity via AMP-production and/or toxic side-effects of
1065 overactivation of the proPO cascade.

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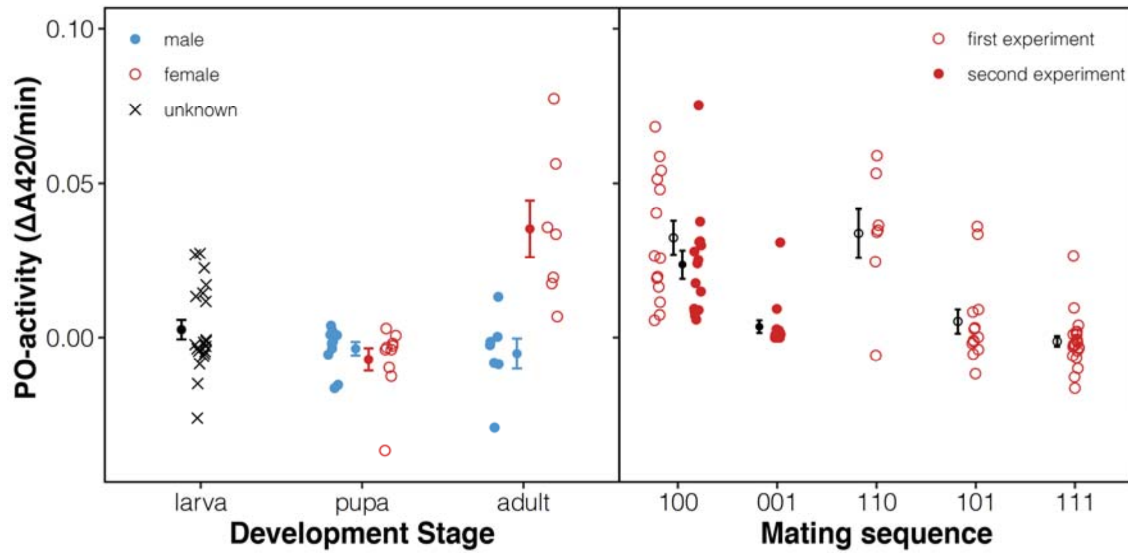

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1068 **Figure 2: Sex-specific regulation of phenoloxidase levels.**

1069 **(a)** There were significant differences in PO activity throughout development, with levels near zero
1070 detected in male (blue) and female (red) pupae and virgin adult males, but detectable levels in
1071 (unsexed = black) larvae and high levels in virgin adult females. **(b)** PO activity measured on day 3 in
1072 females mated only on day one (100), day one and two (110), day one and three (101), or on all days
1073 (111) (open symbols). A second experiment measured PO activity for a random set of females
1074 assigned to treatments 100 and 001 (mated only on day three) (filled symbols). Female PO activity is
1075 reduced after mating but is then quickly restored (compare also to virgin females (i.e. 000 treatment)
1076 in **(a)**. Shown are means ± 1 SE and individual observations. PO-activity was corrected for body mass
1077 by including mass as a covariate in all analyses but is here displayed as raw data since the mean
1078 amount of tissue in samples was similar for all groups.

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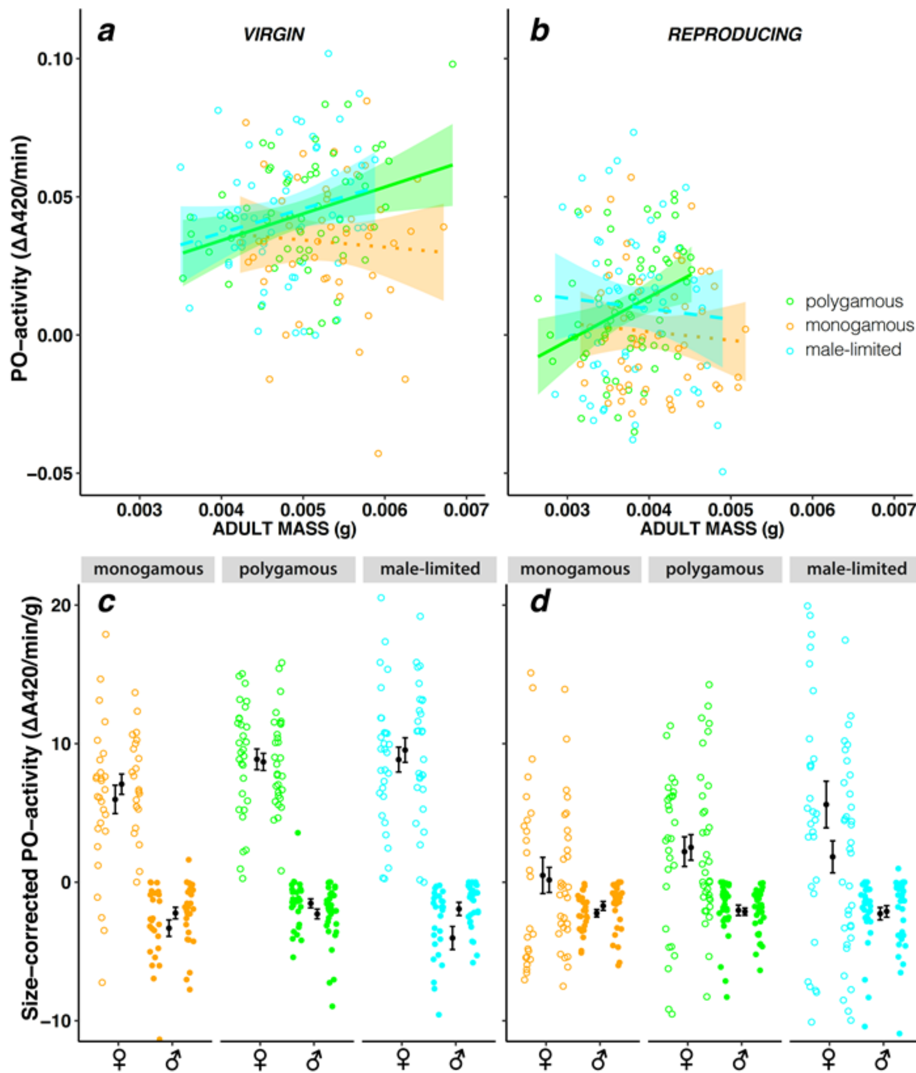

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1081 **Figure 3: Microevolutionary change in PO activity during experimental evolution.**

1082 PO activity measured from whole-body samples of virgin **(a)** and mated **(b)** females from polygamous
1083 (green) monogamous (orange) and male-limited (blue) evolution lines. The mating treatment
1084 significantly reduced female PO activity and male-limited and polygamous females had higher PO
1085 activity than monogamous females. Polygamous and monogamous females also differed significantly
1086 in the relationship between body mass and PO activity, suggesting that different allocation strategies
1087 evolved under the alternative mating regimes. Given are regression slopes, shaded 95% confidence
1088 limits, and individual observations. Males from the regimes did not express detectable levels of PO
1089 activity and showed no significant differences among regimes and mating treatments
1090 (Supplementary Table 1c). In the lower panels, sex differences in size-corrected PO activity is
1091 illustrated in each regime for **(c)** virgin and **(d)** reproducing beetles (means ± 1SE and individual
1092 measures).

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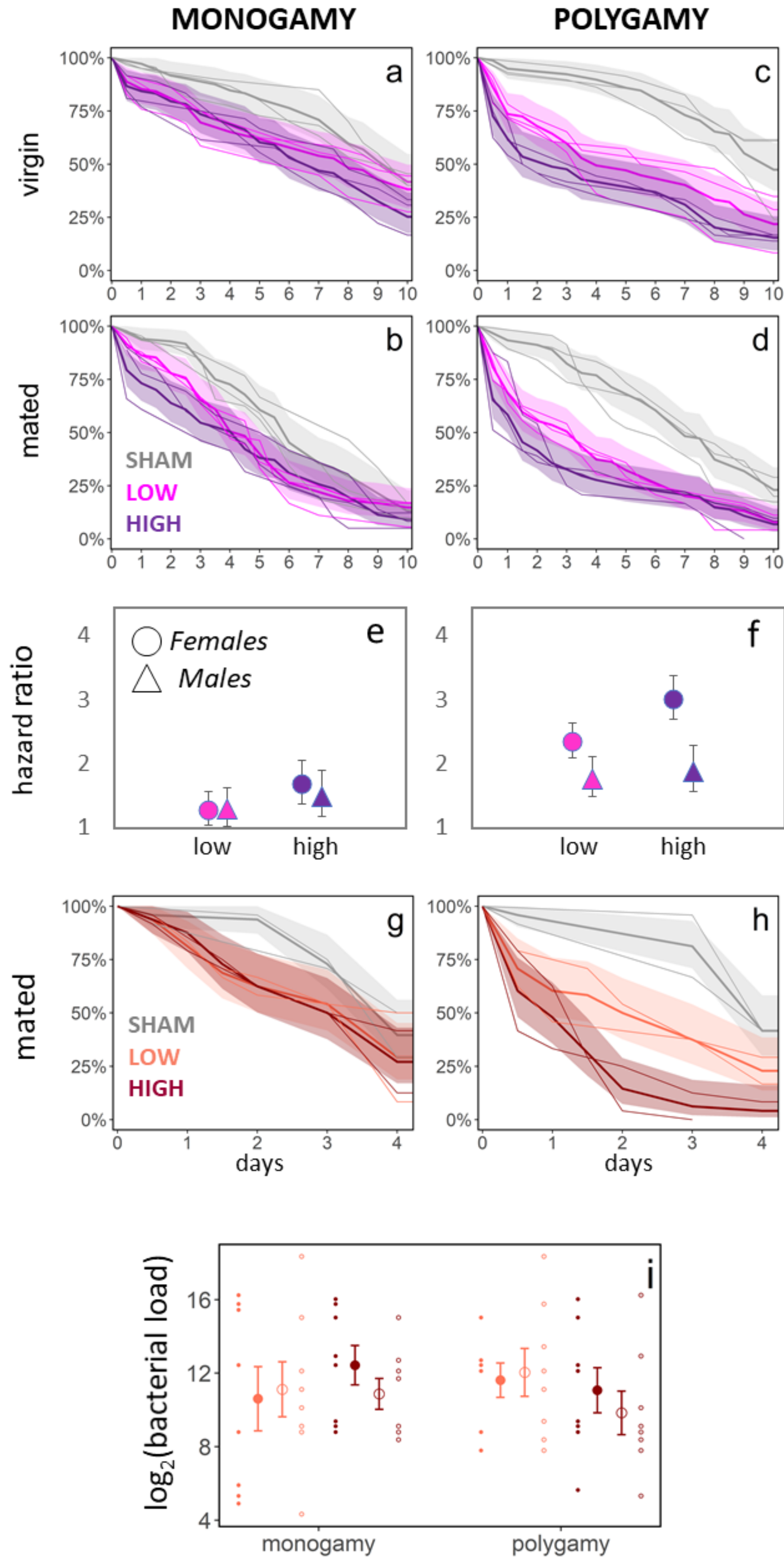

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1096

1097 **Figure 4: Microevolutionary change in tolerance to bacterial infection during experimental**
1098 **evolution under alternative mating regimes.**
1099 Response to bacterial infection was estimated by the change in mortality rate between individuals
1100 infected with two doses of bacteria and a sham control. When infected with the gram-positive
1101 bacteria *B. thuringiensis*, monogamous females **(a, c)** had significantly higher survival under infection
1102 compared with polygamous females **(b, d)**, while virgin **(a, b)** and mated **(c, d)** females had similar
1103 responses. Shown are survival curves for each replicate evolution line (thin lines) together with mean
1104 survival (thick line) and 95% confidence limits (shaded area) based on all three replicate lines per
1105 regime and mating treatment. Virgin males (triangles) from monogamous **(e)** and polygamous **(f)**
1106 regimes did not show the strong differences seen in virgin females (circles), resulting in an apparent
1107 increase in sexual dimorphism in response to infection in the polygamy regime (compare panel **e** and
1108 **f**) (means ± 1SE; lower dose = 1.0 OD, higher dose = 2.0 OD for females and 2.5 OD for males). When
1109 mated females were infected with the gram-negative bacteria, *P. entomophila*, which allowed
1110 assaying of in vivo bacterial counts in infected individuals, monogamous lines **(g)** again showed
1111 higher survival under infection compared with polygamous lines **(h)** (lower dose = 0.5 OD, higher
1112 dose = 1.0 OD). **(i)** Counts of bacterial loads in females 12h post infection showed that difference in
1113 survival were likely not due to more efficient clearance of bacteria in monogamous lines. Means ±
1114 1SE per replicate line (two lines used per regime and dose) and individual estimates per assay.
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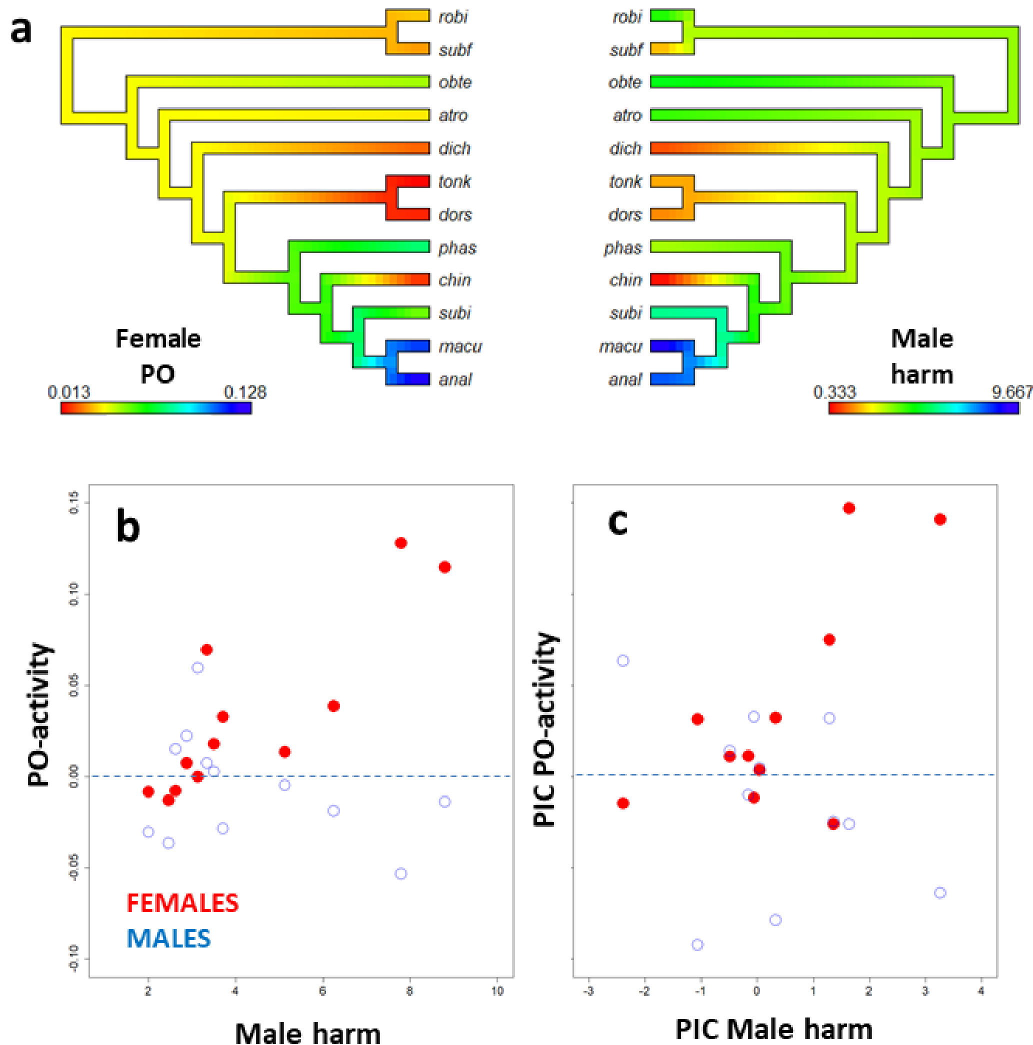

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1118 **Figure 5: Phylogenetic covariance between harmfulness of male genital morphology and**
1119 **PO activity in virgin male and female seed beetles**.
1120 **(a)** Female PO activity and the harmfulness of male genitalia mapped on the phylogeny of the 12
1121 species used. Scores are given by color from blue (high harm/PO) to red (low harm/PO). Lower panels
1122 show correlations across species between male harmfulness and male (blue open) and female (red
1123 closed) PO activity, shown as **(b)** raw tip data and **(c)** phylogenetic independent contrasts (PICs).
1124 Standard errors around each species' mean were typically of the magnitude ~0.02 for male and
1125 female PO activity, and ~0.6 for male genital morphology. The y-axes of **b)** and **c)** are scaled to have
1126 the same range. Species codes represent robi = *Amblycerus robinae*; subf = *Zabrotes subfasciatus*;
1127 obte = *Acanthoscelides obtectus*; atro = *Bruchidius atrolineatus*; dich = *Bruchidius dichrostachydis*;
1128 tonk = *Megabruchidius tonkineus*; dors = *Megabruchidius dorsalis*; phas = *Callosobruchus phaseoli*;
1129 chin = *Callosobruchus chinensis*; subi = *Callosobruchus subinnotatus*; macu = *Callosobruchus*
1130 *maculatus*; anal = *Callosobruchus analis*.

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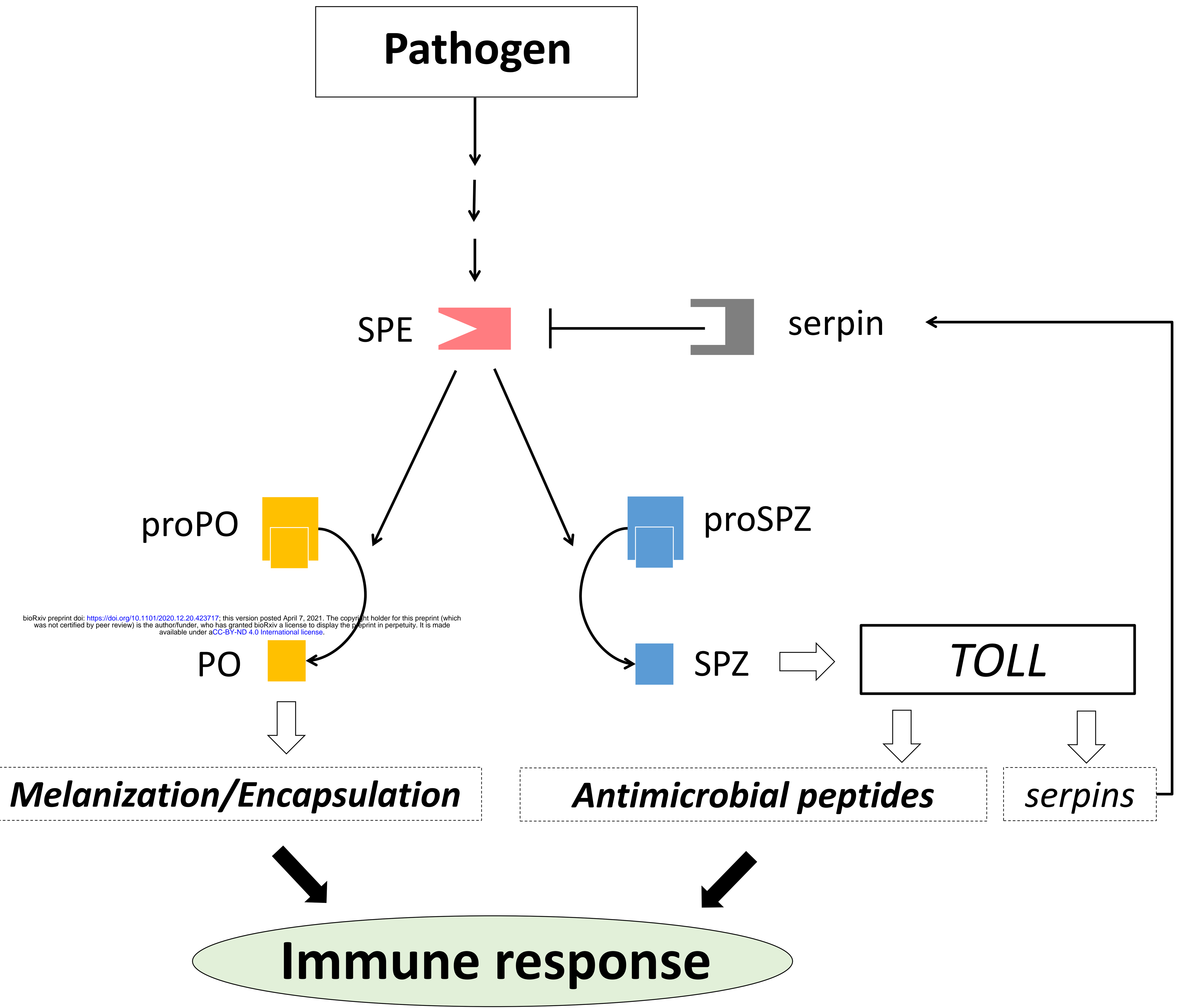

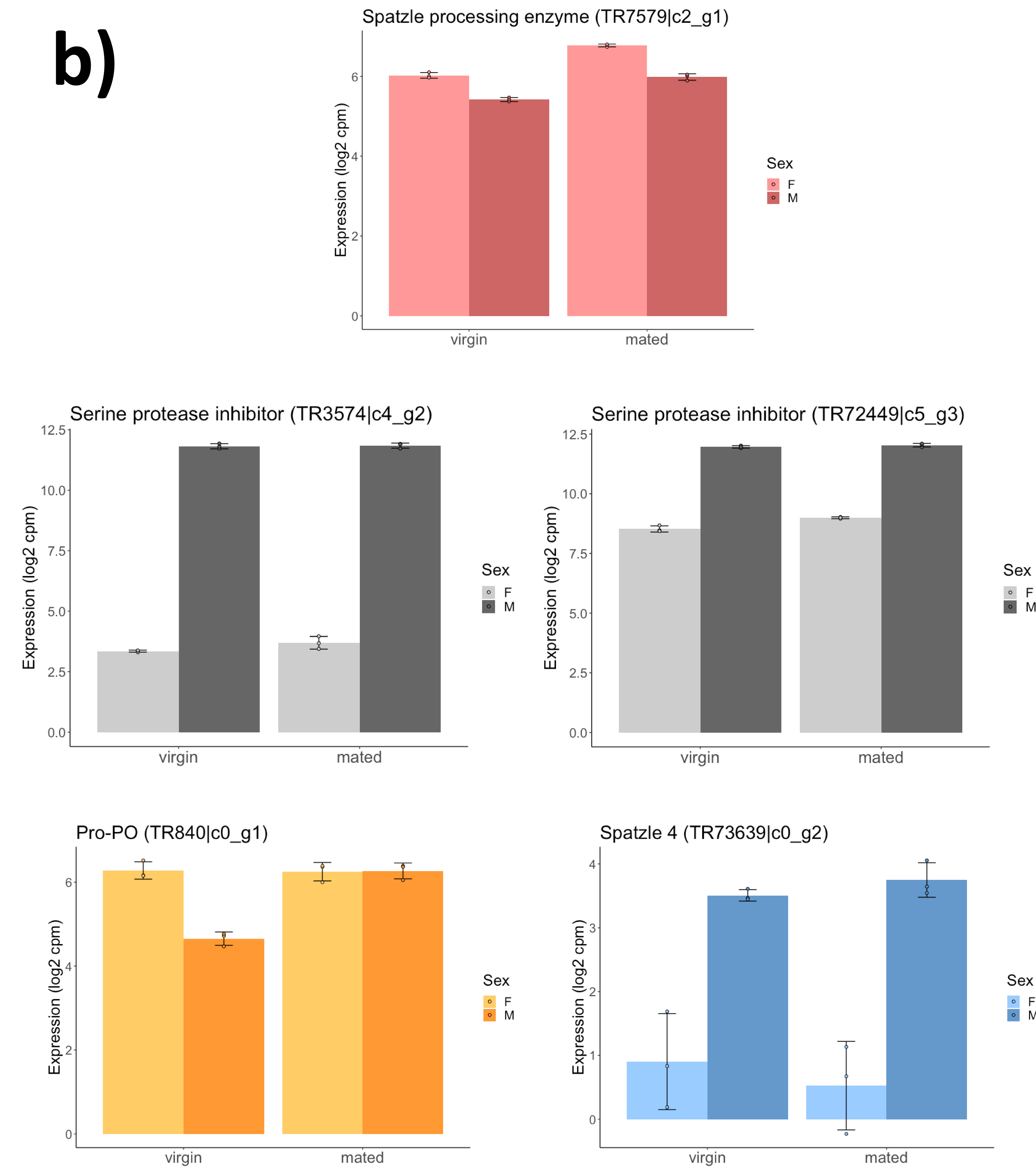

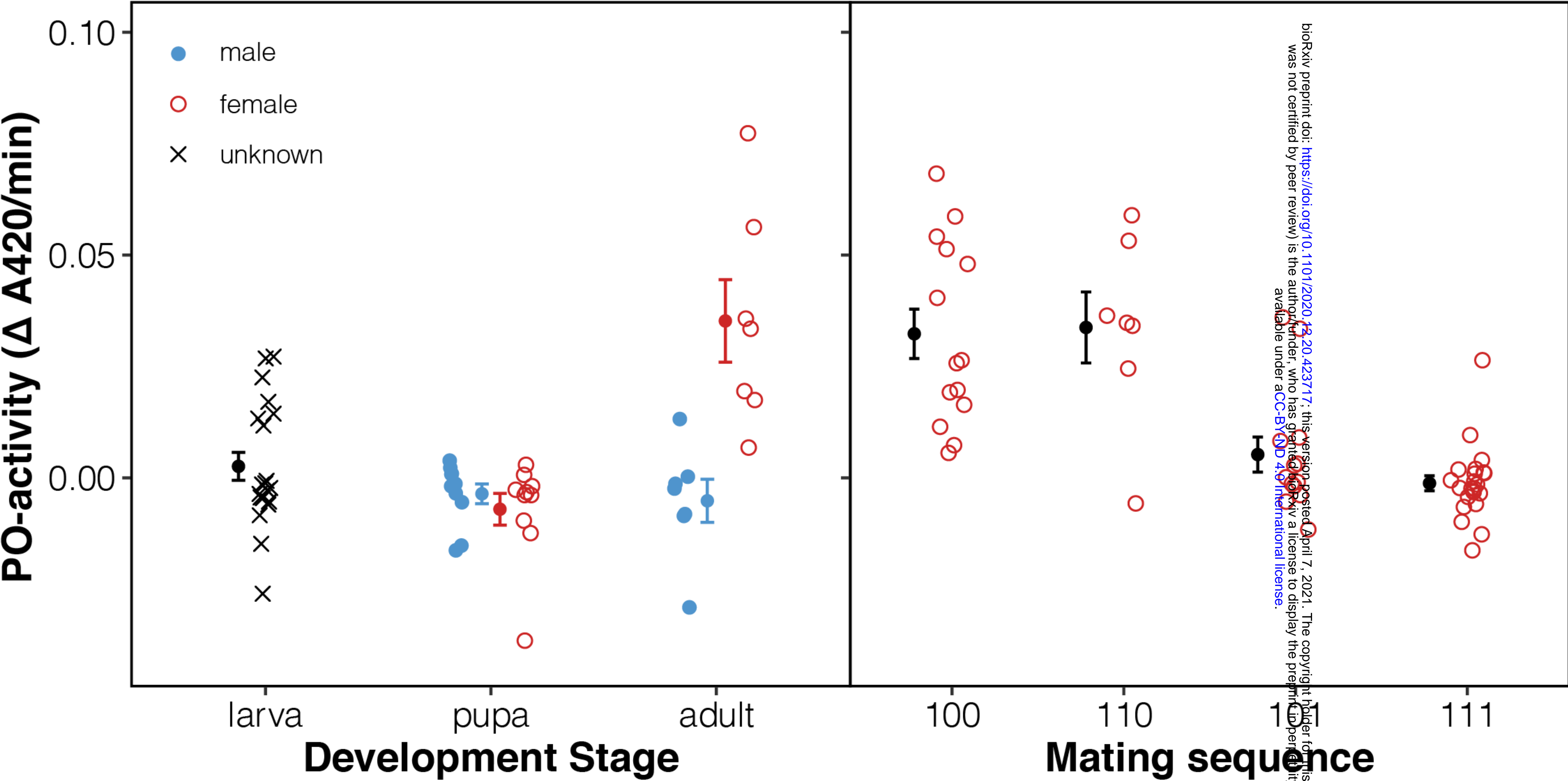

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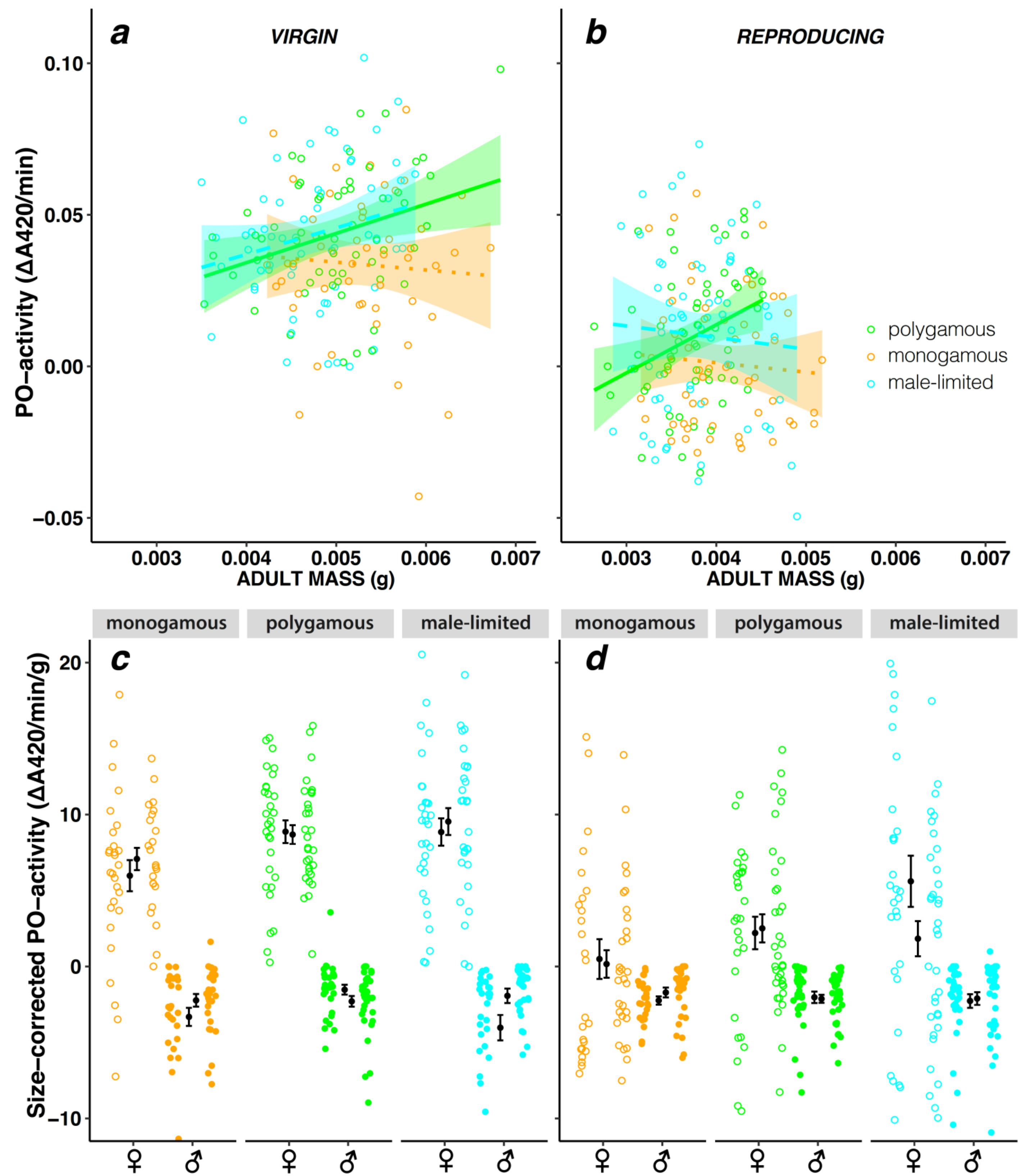

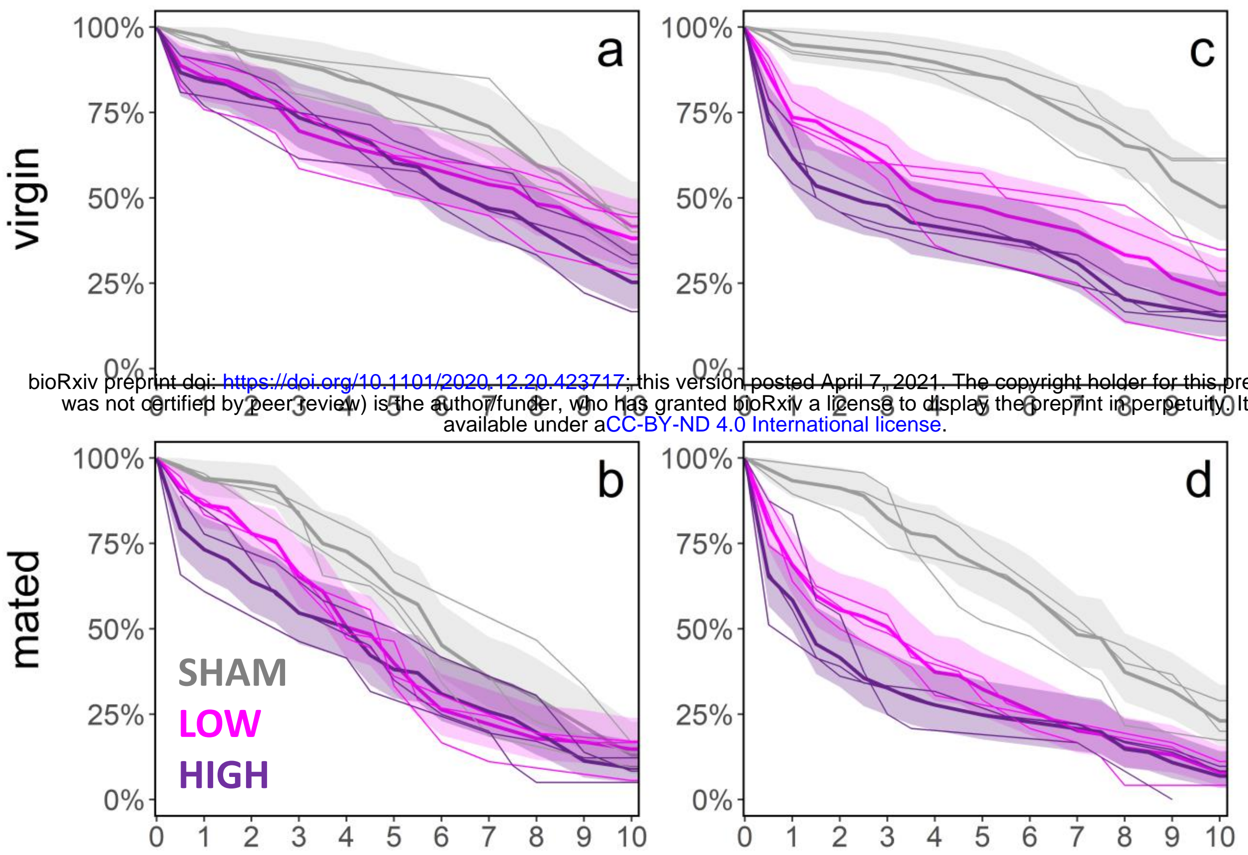

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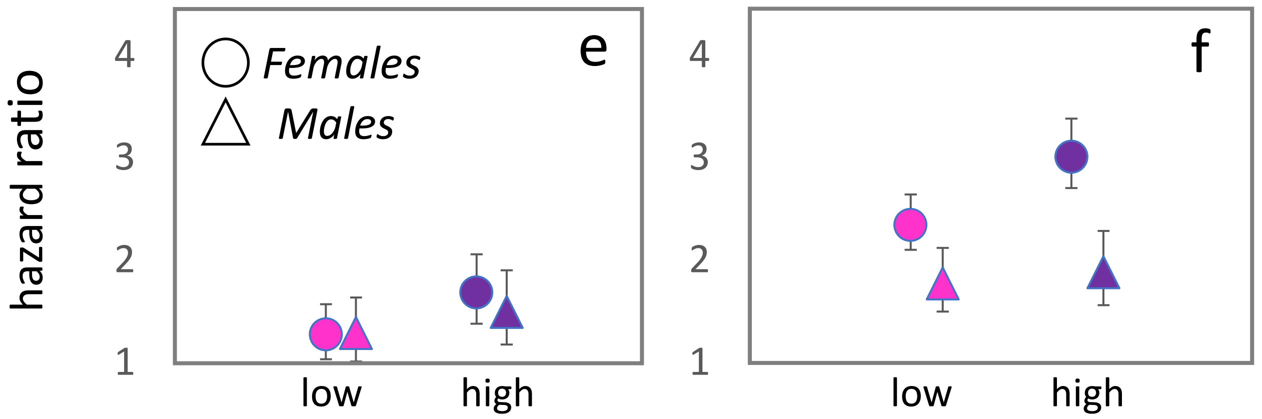

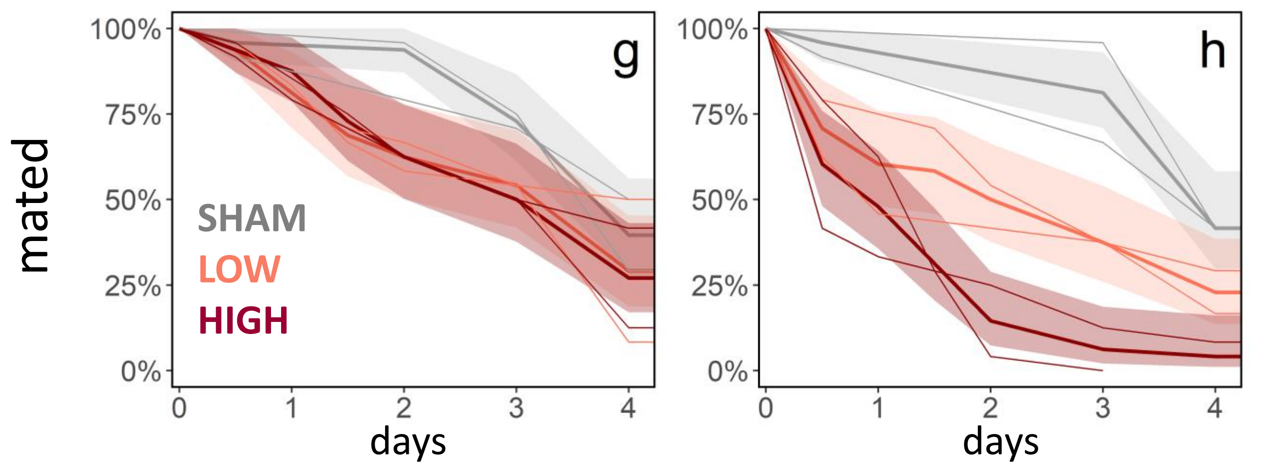

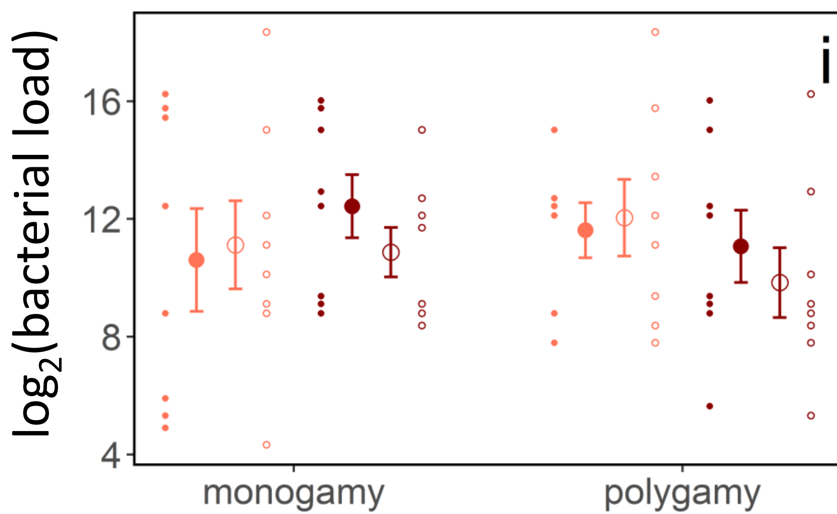

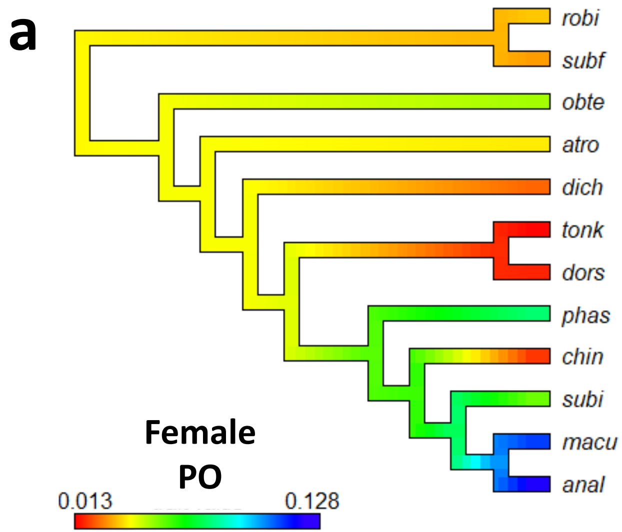
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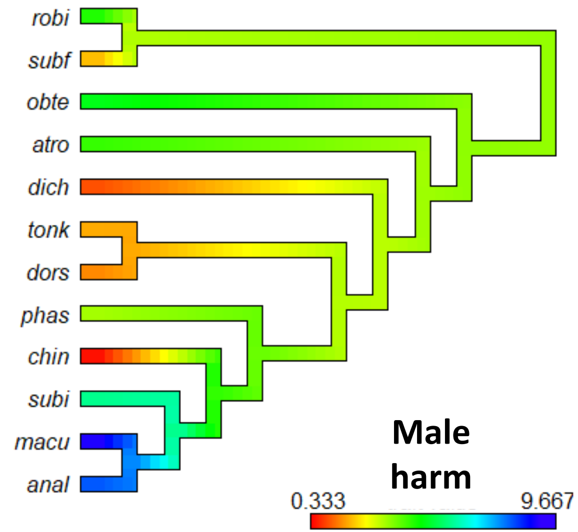
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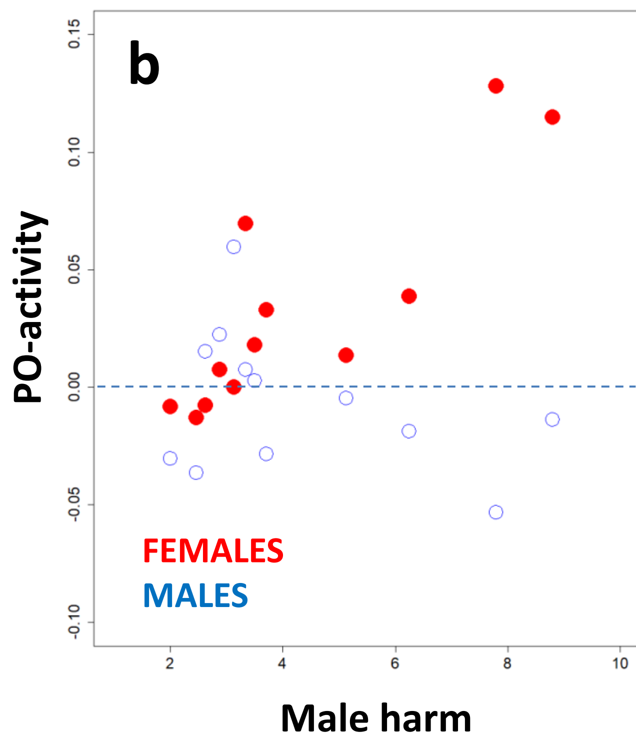
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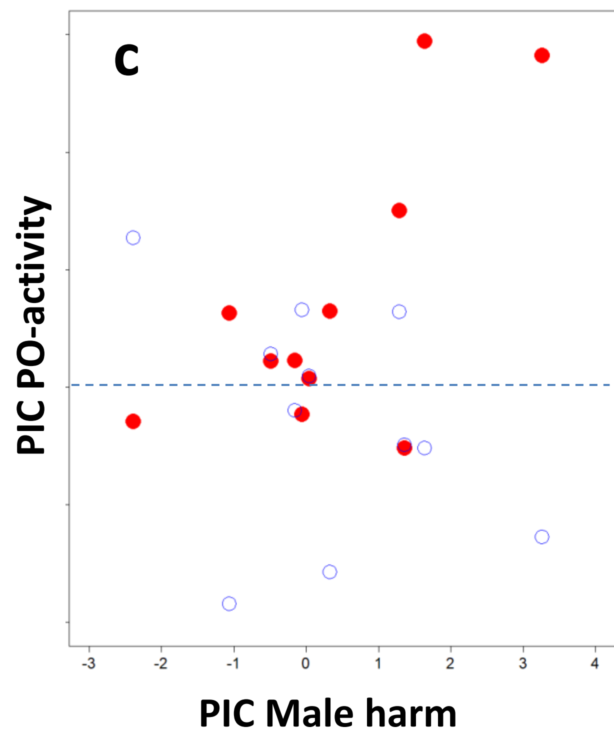
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