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10 **Experimental evidence for sexual selection against inbred males**

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18

Running title: Sexual selection against inbred males

19 **Abstract**

20

21 (1) The detrimental effects of matings between relatives are well known. However, few
22 studies determine the extent to which inbreeding depression in males is due to natural or
23 sexual selection. Importantly, measuring fitness or key fitness components, rather than
24 phenotypic traits allows more accurate estimation of inbreeding depression.

25 (2) We investigate how differences in inbreeding and juvenile diet (i.e. early stressful
26 environment) influence a key component of male fitness, namely their reproductive success.

27 (3) We experimentally created inbred and outbred male mosquitofish (*Gambusia holbrooki*)
28 by mating full-sibs ($f=0.25$). We show that this led to a 23% reduction in genome-wide
29 heterozygosity based on SNPs. Males were raised on different diets early in life to create
30 high-stress and low-stress rearing environments. We then allowed adult males to compete
31 freely for females to test if inbreeding, early diet, and their interaction affect a male's share
32 of paternity.

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33 (4) Early diet had no effect on paternity, but outbred males sired almost twice as many
34 offspring as inbred males ($n = 628$ offspring from 122 potential sires). Using artificial
35 insemination methods we determined that this was unlikely to be due to early embryo
36 mortality of eggs fertilised by inbred males: there was no evidence that male inbreeding
37 status affects the realised fecundity of females ($n=288$).

38 (5) Given there was no difference in male mortality in our competitive mating experiment,
39 the lower reproductive success of inbred males can most parsimoniously be attributed to
40 inbreeding negatively affecting sexually selected traits that affect male mating success
41 and/or sperm competitiveness. We discuss which sexually selected traits might be involved.

42
43 *Keywords:* heterozygosity, inbreeding depression, mosquitofish, paternity, reproductive
44 success

45 **Introduction**

46
47 Environments that are spatially fragmented result in small, isolated populations in which
48 relatives are more likely to mate with each other (Becker et al., 2016, Keller and Waller,
49 2002, Lande, 1988). Mating between relatives often decreases genome-wide heterozygosity
50 in the resultant offspring, which can reduce the mean phenotypic value of traits putatively
51 associated with fitness, so-called 'inbreeding depression' (Falconer and Mackay, 1996, Lynch
52 and Walsh, 1998). Inbred individuals are assumed to be less fit due to greater expression of
53 deleterious, recessive alleles (dominance hypothesis) and/or due to homozygosity at loci
54 where heterozygosity confers an advantage (overdominance) (Charlesworth and
55 Charlesworth, 1987, Charlesworth and Charlesworth, 1999). Traits that are closely related to
56 fitness are predicted to be more likely to show inbreeding depression (DeRose and Roff,
57 1999), because strong directional selection promotes fixation of advantageous alleles, and
58 rapidly eliminates deleterious dominant alleles (DeRose and Roff, 1999, Lynch and Walsh,
59 1998). By measuring traits that are only weakly related to fitness researchers underestimate
60 the true effects of inbreeding on fitness. More studies are needed that directly quantify the
61 effects of inbreeding on fitness or, given the logistic challenges of measuring net fitness,
62 studies that focus on key fitness components (Reed and Frankham, 2003, Hedrick and
63 Kalinowski, 2000, Huisman et al., 2016).

64
65 To date, relatively few experimental studies have looked at the effects of inbreeding on
66 fitness estimates in non-domesticated animals. Of these studies, most focus on female

67 reproductive output, or non-sex-specific life history traits (e.g. Pilakouta and Smiseth, 2016),
68 and only a handful have specifically looked at male fitness. For example, Zajitschek et al.
69 (2009) showed that highly inbred males sire fewer offspring than outbred males; Michalczyk
70 et al. (2010) reported that inbreeding depression reduces sperm competitiveness, which can
71 affect male's fertilization; Konior et al. (2005) estimated the proportion of offspring sired by
72 outbred and inbred males and found that it was lower for outbred males; and Bickley et al.
73 (2013) showed a tendency for inbred males to sire fewer offspring when in direct
74 competition with outbred males.

75
76 Mating success and fertilization success under sperm competition are major determinants of
77 male fitness in most species (Birkhead and Pizzari, 2002, Andersson, 1994, Shuster and
78 Wade, 2003). Sexually selected traits that confer a mating or fertilization advantage are
79 often under strong directional selection and, in addition, they tend to be condition-
80 dependent (Rowe and Houle, 1996, van Oosterhout et al., 2003, Møller, 1993). Condition-
81 dependence has been described as a form of 'genetic capture' because condition reflects how
82 well the individual accumulates resources (Rowe and Houle, 1996, Tomkins et al., 2004). The
83 ability to acquire condition is likely to depend on many traits (e.g. foraging ability, food
84 absorption efficiency) that could be negatively affected by inbreeding. In addition, male-
85 male competition may magnify the effects of inbreeding depression on male reproductive
86 success due to inbred males being weaker competitors or having a poorer ability to obtain
87 territories (Yun and Agrawal, 2014, Meagher et al., 2000, Joron and Brakefield, 2003). It is
88 therefore plausible that, due to sexual selection, male mating success will show greater
89 inbreeding depression than is seen for naturally selected traits that 'capture' less genetic
90 variation. These data cannot, however, be obtained from studies that measure male lifetime
91 reproductive output that confound lifespan (i.e. viability selection) with reproductive
92 success per breeding event (i.e. sexual selection).

93
94 There is high variation in the reported magnitude of inbreeding depression in the available
95 experimental studies of wild animals that try to measure fitness (e.g. Thonhauser et al.,
96 2014, Bickley et al., 2013, Harano, 2011, Meagher et al., 2000). One possible source of
97 variation is whether or not test individuals experience a stressful environment (Armbruster
98 and Reed, 2005, Fox and Reed, 2011). Inbreeding might result in individuals less able to
99 buffer their development against environmental stress (Miller, 1994). Dietary and
100 temperature stress, for example, can increase levels of inbreeding depression (e.g.

101 Dahlgard and Loeschcke, 1997, Kristensen et al., 2008, Auld and Henkel, 2014, Freitak et al.,
102 2014) as can stress arising from intraspecific competition (Meagher et al., 2000, Joron and
103 Brakefield, 2003, Yun and Agrawal, 2014). More generally, rearing animals in a benign lab
104 environment (or plants in greenhouses) is often invoked to explain the absence of
105 inbreeding depression in laboratory studies (Duarte et al., 2003, Enders and Nunney, 2012).
106 Another potential source of variation in estimates of inbreeding depression might be that
107 the evolutionary history of study populations affects the baseline level of heterozygosity. For
108 instance, as mean heterozygosity in a population decreases the difference in heterozygosity
109 between offspring of closely related individuals and those from random matings decreases
110 (Pekkala et al., 2014). This makes it harder to detect inbreeding depression (see also Miller
111 and Coltman, 2014). To date, experimental studies that investigate how these different
112 potential sources of variation influence the effects of inbreeding on fitness-enhancing traits
113 remain scant (but see Dahlgard and Loeschcke, 1997, Reed and Frankham, 2003, Pekkala et
114 al., 2014).

115

116 Here we conduct an experiment to investigate how differences in inbreeding level and
117 juvenile diet (manipulated to create a stressful environment) influence a key component of
118 male fitness, namely reproductive success when competing for mates and fertilization
119 opportunities in the mosquitofish, *Gambusia holbrooki*. *G. holbrooki* is a poeciliid fish
120 endemic to North America, but now introduced worldwide. Mosquitofish are non-migratory,
121 and are often resident in relatively small bodies of water, such as ponds and streams (Pyke,
122 2005). This makes it likely that inbreeding occurs naturally if a few fish become isolated in a
123 small area. There is sufficient genetic variation in our study population for inbreeding to lead
124 to a detectable, and predicted, decline in heterozygosity (see Results). Mosquitofish have
125 internal fertilization and males transfer sperm to females via a modified anal fin called the
126 gonopodium (Pyke, 2005). Males do not court, but perform coercive 'sneak' copulations in
127 which they approach a female from behind and thrust their gonopodium towards her
128 gonopore (Bisazza and Marin, 1995, Langerhans, 2011). Male size is highly variable and small
129 males have greater manoeuvrability that seems to increase their propensity to sneak
130 copulations (Pilastro et al., 1997). Large males are, however, socially dominant and might
131 transfer more sperm per encounter because they have larger sperm reserves (O'Dea et al.,
132 2014). Female size varies considerably and is strongly correlated with fecundity (Bisazza et
133 al., 1989, Callander et al., 2012). Females give birth to live young. Finally, standing variation

134 in heterozygosity is positively correlated with male reproductive success when males
135 compete for mates in experimental ponds (Head et al., 2016).
136
137 We experimentally generated inbred and outbred males that were initially reared on
138 different diets as juveniles (Vega-Trejo et al., 2016a). We then allowed adult males to
139 compete freely for access to females and quantified their share of paternity. The ability to
140 gain paternity under sperm and mating competition is a key male fitness component in
141 species with high levels of female polyandry, such as *G. holbrooki* (Bisazza et al., 2001,
142 Pilastro et al., 1997). Importantly, our experimental design allows us to isolate sexual
143 selection (as opposed to other forms of natural selection) as the mechanism driving any
144 inbreeding depression because we eliminated variation in male mortality. In a second
145 experiment we tested, and confirmed, that being inbred did not affect a male's non-
146 competitive fertilisation ability and/or elevate embryo mortality. We established this by
147 artificially inseminating females with either an inbred or an outbred male's sperm and
148 noting their realised fecundity (i.e. offspring at birth). In addition to the experimental
149 manipulation of inbreeding status using a controlled pedigree we directly estimated each
150 male's genome wide heterozygosity (based on >3000 SNPS) to estimate whether the direct
151 use of an actual estimate of heterozygosity provides a more powerful means to detect
152 inbreeding depression than the binary division of males into inbred and outbred. Our design
153 also allowed us to test the prediction that inbreeding depression for reproductive success
154 would be greater for males reared in a stressful juvenile environment.

155

156 **Methods**

157

158 *Origin and maintenance of fish*

159

160 We used mosquitofish descended from wild caught fish collected in Canberra, Australia. The
161 design that we used to create inbred and outbred males that were then reared on different
162 diets, is fully described in Vega-Trejo et al. (2016a). In brief, in each experimental block we
163 mated individuals from two full sibling families (e.g. A and B in block 1, C and D in block 2
164 and so on). Brothers and sisters from full sibling families were paired to create inbred
165 offspring (AA, BB; $f=0.25$) and outbred offspring with reciprocal male-female crosses (AB,
166 BA) to generate four cross-types. We set up 29 blocks (= maximum of 116 different family
167 pairings types). The 452 male offspring from 192 broods (some experimental blocks had

168 more than one pairing of a given type) were then reared individually in 1L tanks that were
169 distributed randomly throughout a temperature-controlled room (14:10 h photoperiod at
170 28°C). Males underwent a diet manipulation for 21 days from day 7 to 28 post birth that lead
171 to almost zero growth (Vega-Trejo et al., 2016a). Fish on the control diet were fed *ad libitum*
172 with *Artemia* nauplii twice daily (i.e. standard laboratory feeding regime), while fish on the
173 restricted diet were fed 3mg of *Artemia* nauplii once every other day (i.e. < 25% of the
174 control diet). Broods were split evenly between the control and restricted diet.

175

176 *Experimental design - competitive mating scenario*

177

178 To determine whether inbreeding, diet, or their interaction predict paternity we set up
179 mating trials in which four unrelated males, one per treatment, could compete and mate
180 freely with a stock virgin female in a 60L tank (n=31). Males were randomly assigned to each
181 replicate and were not matched for size (size range: 18.51 - 26.96 mm). We have previously
182 shown that inbred and outbred males do not differ in size at maturity (Vega-Trejo et al.,
183 2016a). After a week we removed the female and gave the males a week to recover. The
184 process was then repeated with two more females. The four males in each replicate were
185 kept together for all three trials. The 93 test females were each placed in individual 1L tanks,
186 and we checked twice daily for six weeks whether she had given birth. Offspring were
187 collected immediately and preserved (see below). Adults were euthanized, preserved in
188 absolute ethanol and stored at -20°C.

189

190 *Male morphology*

191

192 All males were measured before we placed them in tanks with females. Males exhibit
193 minimal growth after maturation (Cabral and Marques, 1999, Pyke, 2005, Kahn et al., 2012),
194 so we did not remeasure them between trials. Males were anaesthetized by submersion in
195 ice-cold water for a few seconds to reduce movement, placed on polystyrene with a
196 microscopic ruler (0.1 mm gradation), and photographed. We measured male standard
197 length (SL = snout tip to base of caudal fin) and gonopodium length (intromittent organ
198 modified from the anal fin) using Image J software (Abramoff et al., 2004). The test males
199 were 28 to 37 weeks post-maturity and were marked with a small coloured dot for visual
200 identification using fluorescent elastomer (Northwest Marine Technology, WA) injected
201 subcutaneously behind the caudal fin. They had at least four days to recover before being

202 placed in 60L tanks to start mating trials. We calculated relative gonopodium size as the
203 residuals from a linear regression of gonopodium size (log) on SL (log) (Booksmythe et al.,
204 2016).

205

206 *Paternity analysis*

207

208 To determine male reproductive success and heterozygosity for the fish in our experiment
209 we took tissue samples from each male (n=122), females that bred (n=79 of 93), and up to
210 10 randomly chosen fry per female (n=628 offspring). In total, 39 of 79 females produced 10
211 or fewer fry; and we sampled 72% of the total number of fry born (628 of 878).

212

213 Two of the 124 males (both outbred) were missing at the end of the trial (i.e. escaped or
214 died) and therefore no tissue was available. DNA was extracted from the tail muscle/caudal
215 fin of adults, and from the whole body, excluding the head, of fry. We used Qiagen DNeasy
216 Blood & Tissue extraction kits following the manufacturer's instructions. After extraction,
217 DNA samples were SNP genotyped. Full methods for the paternity analysis are in the
218 Appendix S1 in the Supporting information (see also Booksmythe et al., 2016).

219

220 *Heterozygosity*

221

222 We estimated heterozygosity (H) as the number of SNP loci that were scored as
223 heterozygous divided by the total number of successfully classified loci (L) for each male
224 who was a potential sire in the competitive mating experiments (F_{het}). This is essentially a
225 measure of genome wide heterozygosity. F_{het} is identical to $1 - F_{hom}$ in Bérénos et al. (2016);
226 and to H/L in Szulkin et al. (2010, Table 2), albeit that there are minor differences in L among
227 individual males; $L = 3360 \pm 2.68$ (mean \pm SE) loci per male were successfully classified. We
228 found that a brother-sister mating led to a significant decline in offspring F_{het} ($F_{1,120} = 215.1$,
229 $P < 0.001$) because the proportion of classified loci per male that were heterozygous was
230 0.239 ± 0.025 (mean \pm SD; range: 0.185 - 0.288) in inbred males (n=62) and 0.311 ± 0.028
231 (mean \pm SD; range: 0.263 - 0.378) in outbred males (n=60). The mean heterozygosity of
232 inbred fish was therefore 23.2% less than that of outbred fish, close to the expected 25%
233 decline in F_{het} . We also calculated the mean heterozygosity of the 79 females that bred and
234 of the 628 offspring that were genotyped. For the females, F_{het} was 0.314 ± 0.003 ; and for
235 the offspring, F_{het} was 0.318 ± 0.001 . These values do not differ significantly from that for

236 outbred males (one-way ANOVA: $F_{2,763} = 2.576$, $P = 0.077$, $n = 60$ males, 79 females, 628
237 offspring). There is therefore no detectable sex difference in heterozygosity, and no decline
238 in heterozygosity in the mating trial between outbred individuals in the parental and
239 offspring generation.

240

241 *Experimental design – non-competitive mating scenario*

242

243 To test whether inbred males have lower non-competitive fertilisation success (i.e. whether
244 eggs were fertilised or not) and/or sired offspring with lower embryo survival we artificially
245 inseminated females with a known quantity of sperm from a single male who was either
246 inbred or outbred ($n = 72$ inbred, 72 outbred males; split evenly between high and low food
247 diets) and looked at how many offspring the females gave birth to. If observed, we attribute
248 any difference between the two types of males in the number of offspring born to some
249 eggs not being fertilised and/or embryo mortality. Each male was used to inseminate two
250 females from our lab stock population (n total = 288 females). To inseminate females we
251 first anaesthetised the male in iced water, and stripped his sperm (Matthews et al., 1997).
252 To strip sperm males were placed on their side on a glass slide under a dissecting
253 microscope. The gonopodium was swung forward and 100 μ L of saline solution (0.9%NaCl)
254 was placed on the slide at the gonopodium tip. Gentle pressure was then applied to the
255 abdomen at the base of the gonopodium so that the ejaculate was released into the saline
256 solution. We used a micropipette to transfer 10 intact sperm bundles (in 3 μ L saline solution)
257 directly into the reproductive tract of each of two anaesthetised females. The use of intact
258 sperm bundles results in better fertilisation success than using bundles that have been
259 broken up (Zajitschek et al., 2009). After insemination females were housed individually in
260 1L tanks, which contained a mesh divider and plastic plants. Females were fed and checked
261 for newborn fry twice daily until they gave birth or until 6 weeks had elapsed. We recorded
262 the number of fry born blind to the inbreeding status of the male.

263

264 *Statistical analysis*

265

266 We used Generalized Linear Mixed-effect models (GLMM) with Poisson error to test for
267 fixed effects of inbreeding, diet, body size, relative gonopodium length, and the interaction
268 between inbreeding and diet on how many offspring each male sired. There is no significant
269 effect of inbreeding on relative gonopodium length (GLMM: $\chi^2 = 0.529$; $P = 0.467$; $n = 124$).

270 Consequently, including relative gonopodium length in the model does not mask any effects
271 of inbreeding that might act via an effect on gonopodium length (i.e. it is not a covariate
272 measured post-treatment *sensu* Gelman and Hill (2007,p188)). We used the *glmer* function
273 in the *lme4* package in R 3.0.2 software (R Development Core Team, 2012). As already
274 noted, Heterozygosity (F_{het}) differed greatly between inbred and outbred males. Our main
275 test for whether heterozygosity affects male success under mating competition is therefore
276 simply the effect of inbreeding status. However, to test whether heterozygosity, after
277 controlling for that associated with inbreeding status, explained additional variation in
278 paternity success, we also standardized heterozygosity. We centered F_{het} so that the mean
279 was 0 for each inbreeding treatment (hereafter F^*_{het}). We then ran the final model including
280 F^*_{het} and its interaction with inbreeding status. An interaction would arise if there is a non-
281 linear relationship between F_{het} and paternity success. To account for overdispersion we
282 included individual as a random effect (Harrison, 2014). Following this correction our data
283 was underdispersed (dispersion parameter = 0.33) and conservative. We included mating
284 trial tank as a random effect to account for potential non-independence. We also included
285 sire and dam identity as random effects in the final model, even though they explained
286 almost no variation in male reproductive success. This can partly be attributed to low
287 statistical power to detect additive genetic variation underlying male reproductive success
288 as, for example, of the 60 sires that provided sons we used in the competitive mating trials,
289 the mean number of sons per sire was 2.07 (range 1-6). All fixed model terms were tested
290 for significance using the Anova function in the *car* package specifying Type III Wald chi-
291 square tests. We removed non-significant interactions following Crawley (2005). All tests are
292 two-tailed and alpha is set at 0.05.

293

294 To test whether females that were artificially inseminated by inbred males produced fewer
295 broods than those inseminated with sperm from outbred males we used a GLMM with
296 Binomial error. Whether or not a female produced a brood (i.e., 0, 1) was the response
297 variable. Inbreeding status, diet, and their interaction were included as fixed factors. We
298 included male identity as a random effect to correct for repeated measurements. We also
299 tested whether male inbreeding status influenced how many fry a female gave birth to. To
300 do so, we used the mean number of fry produced by females (excluding those that did not
301 breed) for each male as the response variable in a GLM with a quasipoisson error structure
302 to account for overdispersion. Male inbreeding status, diet, and their interaction were

303 included as fixed factors. We again removed non-significant interactions following Crawley
304 (2005).

305

306 To estimate the standardized difference among means we calculated Hedges' g following
307 Rosenberg et al. (2013). By convention we refer to $r=0.1$, 0.3 , and 0.5 as small, medium, and
308 large effect sizes respectively (Cohen, 1988).

309

310 Results

311

312 *Male reproductive success under a competitive mating scenario*

313

314 On average, outbred males sired significantly more offspring than inbred males (Table 1, Fig.
315 1). Outbred males sired 6.37 ± 0.88 offspring while inbred males sired 3.76 ± 0.73 (mean \pm
316 SE). This is equivalent to a medium-large effect size of Hedge's $g = 0.41$. More heterozygous
317 males therefore had significantly greater reproductive success.

318

319 *Heterozygosity controlling for inbreeding status*

320

321 We did not find any significant difference in how F_{het}^* affected male reproductive success
322 between inbred and outbred males ($F_{\text{het}} \times \text{inbreeding}$, $\chi^2 = 0.873$; $P = 0.350$). There was also
323 no significant effect of F_{het}^* on male reproductive success (Table 1). Together these findings
324 indicate that the residual variation in heterozygosity (i.e. F_{het}^* in outbred males) did not
325 predict variation in male reproductive success. We also tested whether a GLMM using F_{het}
326 was a better predictor of male reproductive success than a GLMM using inbreeding status
327 (the other fixed model terms: diet, body size, relative gonopodium length, and an
328 interaction between diet and inbreeding status or F_{het}). The amount of variation explained
329 was identical ($R^2 = 0.117$), which confirms that in the analysis using inbreeding status and
330 F_{het}^* , the extra information from the use of actual heterozygosity estimates did not allow us
331 to explain significantly more variation that obtained based solely on the difference in
332 heterozygosity generated by the creation of inbred and outbred males.

333

334 *Diet*

335

336 We did not find an effect of paternal juvenile diet on the number of offspring sired (Table 1).
337 There was also no significant interaction between inbreeding status and diet (GLMM= $\chi^2 =$
338 0.297; $P = 0.586$). The effects of inbreeding were therefore not exacerbated by juvenile diet.

339

340 *Male morphology*

341

342 Males with a relatively longer gonopodium sired significantly more offspring (Table 1). We
343 did not, however, find an effect of male body size on the number of offspring sired (Table 1).

344

345 *Male reproductive success under a non-competitive mating scenario*

346

347 The inbreeding status of males did not affect how many of the females that we artificially
348 inseminated produced offspring, regardless of which diet the males were reared on (Table
349 2). 48 of 144 females inseminated by an inbred male produced offspring, and 47 of 144
350 females inseminated by an outbred male produced offspring. Likewise, male inbreeding
351 status did not affect the average number of offspring per brood for females that did breed.
352 Outbred males sired 2.86 ± 0.22 offspring while inbred males sired 3.31 ± 0.25 (mean \pm SE;
353 Table 3). There is therefore no evidence that higher early juvenile mortality is biasing our
354 estimate of the share of paternity gained by inbred males downward (i.e. that they fertilized
355 eggs but the offspring died before being counted at birth).

356

357 **Discussion**

358

359 Inbreeding is expected to lower fitness due to the negative effects of decreased
360 heterozygosity (Charlesworth and Charlesworth, 1987, Lynch and Walsh, 1998). Here we
361 used a controlled breeding design combined with a genome wide SNP-based measure of
362 heterozygosity to test whether inbreeding, as well as residual variation in heterozygosity,
363 affects a key component of male fitness, namely reproductive success when males compete
364 for fertilization opportunities. We found that one generation of inbreeding between full-
365 siblings ($f=0.25$), leading to a 23.2% decline in the proportion of SNP loci that were
366 heterozygous, significantly decreased paternity success (6.37 vs 3.76 offspring per male).

367

368 Outbred males sired significantly more offspring than inbred males when they had to
369 compete for mates and fertilization. This result cannot be attributed to viability selection as

370 only two of 124 males died during the mating trials, and both were outbred. In addition, our
371 artificial insemination study of singly-mated females showed that a male's inbreeding status
372 did not affect the likelihood that a female bred, or the number of offspring produced per
373 brood. Inbred males are therefore unlikely to have had a lower estimated share of paternity
374 in our competitive mating trials due to higher embryo mortality, or a naturally selected
375 effect due to lower non-competitive fertilization ability. Outbred males therefore appear to
376 be favoured when there is sexual selection. Relative gonopodium length, which is not
377 affected by inbreeding, explained some of the remaining variation in reproductive success in
378 a competitive scenario. Males with a longer gonopodium were significantly more successful.
379 We found no evidence that diet or body size affect male reproductive success. Nor did we
380 find any effect of residual variation in heterozygosity once we accounted for the decline in
381 heterozygosity associated with inbreeding in our pedigree design (i.e. the effect of sires'
382 inbreeding status).

383

384 *Heterozygosity and male fitness*

385

386 There is indirect evidence from correlational field studies that inbreeding reduces male
387 reproductive success (Frère et al., 2015, Cain et al., 2014, Chapman and Sheldon, 2011,
388 Huisman et al., 2016). In contrast, studies comparing the reproductive output of
389 experimentally created inbred and outbred males have yielded less consistent results. For
390 example, inbreeding depression had no effect on offspring production under a non-
391 competitive scenario in male wild house mice and male flour beetles (Michalczyk et al.,
392 2010, Meagher et al., 2000), while the proportion of offspring sired by inbred males was
393 lower than that of outbred males in bulb mites (*Rhizoglyphus robini*; Konior et al., 2005). In
394 guppies (*Poecilia reticulata*), inbred males sired significantly fewer offspring than outbred
395 males, but only when the inbreeding coefficient was at least $f=0.375$ (i.e. two successive
396 generations of full-sib breeding; Zajitschek et al., 2009). Inbreeding is, in essence, simply a
397 process that decreases heterozygosity, which is why heterozygosity is used as a proxy for
398 inbreeding (Miller and Coltman, 2014, Béréños et al., 2016). Our experiment reveals a
399 significant heterozygosity-fitness correlation (HFC) for male *G. holbrooki*. However, we also
400 show that detecting this HFC could be difficult using standing variation in heterozygosity, as
401 occurs in field studies (Coltman and Slate, 2003, Szulkin et al., 2010, Chapman et al., 2009).
402 Specifically, we found no effect of residual heterozygosity (F^*_{het}) on reproductive success for
403 either inbred or outbred males. The variance in (residual) heterozygosity of outbred males in

404 our study should be equivalent to that of males in the field population. (The only caveat is
405 that the variance in heterozygosity in males in the field will be greater if there is inbreeding
406 in the wild. The extent of any difference in heterozygosity will increase with the natural rate
407 of occurrence of inbreeding. We specifically eliminated any such inbreeding in our study by
408 always pairing unrelated fish to create outbred males (Miller and Coltman, 2014, Szulkin et
409 al., 2010)). It is therefore intriguing that in a new study of field-caught males, albeit with a
410 larger sample ($n = 240$ putative sires), we detected a significant HFC for male reproductive
411 success when males competed for females in 24 semi-natural pools (Head et al. unpublished
412 data). One interpretation of this difference in the reported effect of heterozygosity is that
413 when males develop under natural field conditions this exacerbates inbreeding depression
414 (see Throver and Hard, 2009). Another possibility is that there is actually considerable
415 variation in the relatedness of mates in the field, which elevates variation in heterozygosity.
416 This source of variation was eliminated in our study due to the controlled breeding design.
417 That is, F_{het}^* is heterozygosity after removing effects of parents mating with close relatives.
418
419 Studies of inbreeding in the wild generally fail to tease apart natural and sexual selection
420 against inbred males. Reports of lower reproductive success for less heterozygous (i.e.
421 inbred) males could be due to natural selection because of lower rates of survival (e.g.
422 Mulard et al., 2009, Velando et al., 2015, Frommen et al., 2008), which will, all else being
423 equal, reduce their lifetime reproductive success; and/or because inbred males are less
424 attractive to females (including discrimination at the gametic level; Crean and Bonduriansky,
425 2014) or are weaker mating or sperm competitors (Aspi, 2000, Meagher et al., 2000, Joron
426 and Brakefield, 2003, Okada et al., 2011). However, sperm traits may not always be affected
427 by inbreeding depression (Mehlis et al., 2012, Opatová et al., 2016). In our experiment, we
428 can eliminate natural selection through mortality as a major source of variation in male
429 reproductive success (the two male deaths reduce our estimate of inbreeding depression).
430 We can also rule out an effect of male inbreeding status on embryo mortality. When we
431 artificially inseminate virgin females using the sperm of a single male, inbred and outbred
432 males produced the same number of offspring. This finding is similar to studies that have
433 found that the effects of inbreeding depression are not evident under a non-competitive
434 mating scenario (e.g. Meagher et al., 2000, Michalczyk et al., 2010). Sexual selection is
435 therefore the most likely explanation for the lower reproductive success of inbred males.
436 Indeed, by definition, it is the only explanation (aside from Type 1 error) if sexual selection is
437 broadly defined as variation in reproductive success arising from competition for gametes. It

438 should be noted, however, that competitive interactions in the wild might lead to natural
439 selection on traits that indirectly affect the expression of sexually selected traits (e.g. due to
440 trade-offs in investment) and thereby amplify inbreeding depression on traits under sexual
441 selection.

442
443 An obvious question to ask is: what traits account for sexual selection against inbred male *G.*
444 *holbrooki*? Interestingly, in another study we did not detect inbreeding depression in *G.*
445 *holbrooki* for sperm traits (velocity and sperm count) or for male attractiveness (based on
446 two-choice association tests), despite much larger sample sizes than in the current study (J.
447 Marsh et al. unpublished data; data and analysis available on request). The lack of
448 inbreeding depression in sperm traits could be attributed to low genetic variation due to
449 founder effects (Ayres et al., 2010) because *G. holbrooki* are an introduced feral pest species
450 in Australia. Low genetic variation reduces the magnitude of the difference in heterozygosity
451 between inbred and outbred males. However, the inbreeding depression we report here for
452 actual reproductive success makes this a weak explanation. Ultimately, the results we
453 present here highlight the need to look at how inbreeding affects key fitness components,
454 and not only look at phenotypic traits (such as sperm count) that are only indirect proxies
455 for fitness. Based solely on sperm velocity and sperm count, we would not predict a decline
456 in the fertilization ability of inbred males. Of course, inbred males might not have less
457 competitive ejaculates. They might simply be less successful at initially inseminating females.
458 In a separate study we used artificial insemination, controlling for sperm number, to test
459 whether inbred males have less competitive ejaculates than outbred males (J. Marsh et al.
460 unpublished data). There is evidence that the greater the difference in heterozygosity
461 between two competing males the higher the share of paternity gained by the more
462 heterozygous male, suggesting that inbred males will, on average, have less competitive
463 ejaculates.

464
465 *Inbreeding depression in stressful and benign environments*

466
467 Inbreeding depression tends to be higher in a more stressful environment (Armbruster and
468 Reed, 2005, Fox and Reed, 2011). By definition a more stressful environment is one that
469 reduces fitness relative to a baseline environment (Armbruster and Reed, 2005). Our low
470 food diet resulted in almost zero growth over a three-week period (see Vega-Trejo et al.,
471 2016a), which strongly suggests that we created a stressful environment. Corroborating this,

472 we have previously shown that this diet significantly reduces male attractiveness measured
473 as female association time (Kahn et al., 2012). It should, however, be noted that in our
474 current study a low food diet did not reduce a male's ability to gain paternity when
475 competing for mates. Studies of other taxa, mainly insects, show that a poor juvenile diet
476 can reduce the ability of males to gain paternity (e.g. Moreau et al., 2007). This is mainly
477 attributed to a lower sperm count and reduced sperm competitiveness (Rahman et al., 2013,
478 Muller et al., 2015). Elsewhere we have shown that, controlling for age, a poor juvenile diet
479 reduces sperm reserves and sperm replenishment rates in younger male *G. holbrooki* (Vega-
480 Trejo et al., In Press). The males in our current experiment were, however, sufficiently old
481 (28- 37 weeks post-maturation) that juvenile diets should not have affected sperm
482 production. If sperm number is a major determinant of male reproductive success this
483 would partly explain why there was no main or interactive effect of diet on male success.
484 Again, however, this raises the question of the proximate mechanism causing inbred males
485 to have lower paternity.

486

487 Studies of a range of taxa report a weak or no relationship between inbreeding depression
488 and the level of dietary stress (effect size $r = -0.13$ to 0.02 ; Fox et al., 2011, Reed and Bryant,
489 2001, Reed et al., 2003), but most of the focal traits measured in the primary studies are
490 naturally selected. Sexually selected traits that affect male reproductive success are
491 predicted to be more sensitive to inbreeding depression because of their tight links with
492 fitness (Mallet and Chippindale, 2011, Drayton et al., 2007, Tomkins et al., 2004, Bolund et
493 al., 2010), and their greater sensitivity to environmental stress because they tend to be
494 condition-dependent (David et al., 2000, Ingleby et al., 2010). It is therefore intriguing that
495 we found significant inbreeding depression for male reproductive success, but no effect of
496 diet. It is possible that we did not find a dietary effect because the stressful environment
497 was simply not stressful enough or because it was only experienced early in life. More
498 generally, we suggest that studies of many more taxa are needed to establish whether
499 sexually selected traits show the same pattern as naturally selected traits (Armbruster and
500 Reed, 2005, Fox and Reed, 2011) with respect to whether a more stressful environment
501 elevates inbreeding depression.

502

503 *Morphological predictors of male fitness*

504

505 Males with a relatively long gonopodium for their body size had significantly higher
506 reproductive success in a competitive mating scenario, even taking into account the effects
507 of inbreeding and residual heterozygosity. This corroborates results from another study of *G.*
508 *holbrooki* in 24 semi-natural pools (Head et al. unpublished data). Several studies of poeciliid
509 fishes have reported a positive correlation between relative gonopodium length and male
510 fitness (Brooks and Caithness, 1995, Langerhans et al., 2005, Devigili et al., 2015, Head et al.,
511 2015, but see Booksmythe et al., 2016). On the other hand male body size, which is often
512 implicated in sexual selection in *G. holbrooki*, had no effect on reproductive success.
513 Previous studies have found mixed results for the effects of male body size (e.g. small male
514 advantage Pilastro et al., 1997, large male advantage Booksmythe et al., 2013, O'Dea et al.,
515 2014) and we suggest that further studies should look into the potential environmental and
516 social factors that might influence this relationship.

517

518 *Conclusions*

519

520 We conducted an experiment that showed that inbreeding reduces a key fitness component
521 (share of paternity) of male *Gambusia holbrooki*. Our design removed most sources of
522 natural selection (e.g. offspring and adult survival), and our artificial insemination
523 experiment revealed no effect of male inbreeding on embryo mortality, so the lower
524 reproductive success of inbred males strongly suggests that inbreeding affects sexually
525 selected traits. This is important as sexual selection against inbred males could reduce the
526 genetic load (Enders and Nunney, 2012). If inbred males are less likely to mate and/or
527 fertilize eggs, this will reduce the frequency of deleterious recessive alleles and could
528 potentially lower the risk of extinction in small populations (Whitlock, 2000, Radwan et al.,
529 2004, Sharp and Agrawal, 2008, Hollis et al., 2009). This possibility, if generally true in other
530 taxa, could be profitably incorporated into models of population viability, as inbreeding can
531 shape the evolution of key life history traits (Charpentier et al., 2007). Of course, we readily
532 acknowledge that our estimate of the effect of inbreeding on males is based on reproductive
533 success in a specific context (four males competing for a female). This is not an unnatural
534 situation given the wide range in adult sex ratios seen in the field (e.g. Donald, 2007,
535 Cameron, 2004), but the strength of sexual selection might change when there is a less
536 male-biased sex ratio (but see Henshaw et al., 2016).

537

538 Our study is a reminder that standing variation in heterozygosity plays an important role in
539 the likelihood of detecting inbreeding depression in correlational studies. This consideration
540 appears to explain variation in reported levels of inbreeding depression, and heterozygosity-
541 fitness correlations (HFC), in other studies (e.g. Coltman and Slate, 2003, Szulkin et al., 2010,
542 Chapman et al., 2009). Residual variation in heterozygosity, hence the use of HFC, was
543 insufficient to detect inbreeding depression in our study: there was no effect of relative
544 heterozygosity (F^*_{het}) on paternity. We only detected inbreeding depression because our
545 breeding design created males with 23% lower than average heterozygosity. Finally, we have
546 to acknowledge the weakness of measuring fitness components in the laboratory.
547 Nonetheless, there is clearly merit in taking an experimental (hence often lab-based) rather
548 than correlational approach to estimate the magnitude of inbreeding depression:
549 experimentally manipulating inbreeding can eliminate the risk of unmeasured confounding
550 factors, that covary with mating partner relatedness, biasing estimates of inbreeding
551 depression (Becker et al., 2016, Reid et al., 2008). The ideal study, of course, would
552 experimentally create inbred and outbred males, release them into the wild and then
553 monitor their reproductive success while controlling for natural selection (i.e. mortality).
554 Such studies have, however, to the best of our knowledge not yet been conducted (but see
555 Jimenez et al., 1994, Schwartz and Mills, 2005).

556

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558

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564

565 **Data Accessibility:** All data associated with this study have been deposited in the Dryad
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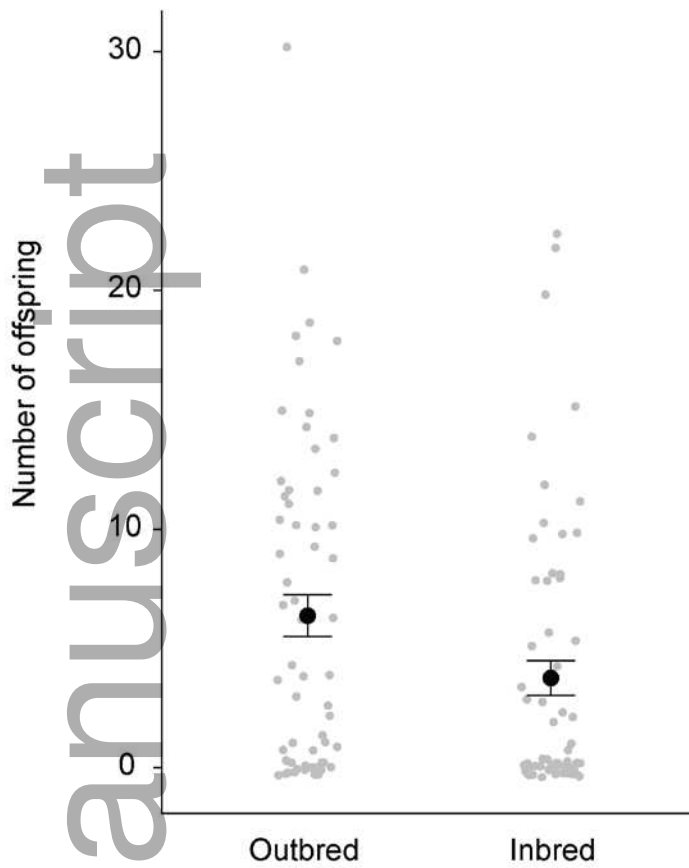
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833

834 Figure 1. Mean number of offspring (\pm SE) sired by outbred and inbred males (n = 122 males
835 genotyped; 60 outbred and 62 inbred). Raw data are represented by dots.

836 Table 1. Results from the mixed model with parameter estimates and chi square (χ^2) tests
837 for heterozygosity, inbreeding, food treatment, size, and relative gonopodium size (residuals
838 of the log-log regression of gonopodium length on body size) on the number of offspring
839 males sired. P-values in bold indicate significant values (n = 628 offspring genotyped).

840

	Predictor	Estimate	SE	χ^2	P
Number of offspring	Intercept	-17.295	13.888	1.551	0.213
	Relative heterozygosity (F^*_{het})	0.114	0.201	0.319	0.572
	Inbreeding (inbred)	-0.943	0.399	5.596	0.018
	Diet (low food)	0.763	0.469	2.643	0.104
	Size [ln(mm)]	12.829	10.004	1.645	0.199
	Relative gonopodium size (residuals)	0.483	0.212	5.179	0.023

Individual identity	3.498
Dam identity	0
Sire identity	0
Mating trial tank	0

841

842 Table 2. Results from the mixed model with parameter estimates and chi square (χ^2) tests
 843 for inbreeding and food treatment on whether the females that we artificially inseminated
 844 produced offspring (n = 288 females).

845

	Predictor	Estimate	SE	χ^2	P
Number of females that produced broods	Intercept	-0.559	0.217	6.616	0.010
	Inbreeding (inbred)	0.145	0.243	0.356	0.551
	Diet (low food)	0.318	0.244	1.696	0.193
	Male identity	0.034			

846

847 Table 3. Results from the generalized linear model with parameter estimates and t tests for
 848 inbreeding and diet treatment on the average number of offspring per brood when females
 849 were inseminated by a single male who was either inbred or outbred (n = 95 females).

850

	Predictor	Estimate	SE	t	P
Number of offspring	Intercept	3.452	0.120	28.858	<0.001
	Inbreeding (inbred)	0.140	0.132	1.064	0.290
	Diet (low food)	-0.177	0.132	-1.344	0.182

851

Authors' contributions

852

R.V.T., M.L.H., and M.D.J. designed the study. R.V.T carried out the experimental work. J.S.K.

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analysed the paternity data. R.V.T., M.L.H., and M.D.J. analysed the data and wrote the

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manuscript. All the authors contributed substantially to revisions, and gave final approval for

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publication.