

1 **Experimental evidence for sexual selection against inbred males when it truly counts**

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3 Regina Vega-Trejo<sup>1,\*</sup>, Megan L. Head<sup>1</sup>, J. Scott Keogh<sup>1</sup> and Michael D. Jennions<sup>1</sup>

4 <sup>1</sup>Division of Evolution, Ecology and Genetics

5 Research School of Biology

6 The Australian National University

7 Acton, Canberra

8 ACT 2601

9 Australia

10

11 \*Corresponding author; Regina Vega-Trejo, e-mail: [reginavegatrejo@gmail.com](mailto:reginavegatrejo@gmail.com)

12 Ph: +61 2 6125 3828

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15 **Abstract**

16

17 Although there are many correlational studies, unbiased estimates of inbreeding depression  
18 only come from experimental studies that create inbred and outbred individuals. Few such  
19 studies determine the extent to which inbreeding depression in males is due to natural or  
20 sexual selection. Importantly, traits that are closely related to fitness are predicted to be  
21 most strongly affected by inbreeding depression, so measuring fitness or key fitness  
22 components, rather than phenotypic traits, is necessary to estimate inbreeding depression  
23 accurately. Here, we experimentally created inbred and outbred male mosquitofish  
24 (*Gambusia holbrooki*) by mating full-sibs ( $f=0.25$ ). We show this led to a 23% reduction in  
25 genome-wide heterozygosity. Males were then raised on different diets early in life. We  
26 then allowed adult males to compete freely for females to test if inbreeding, early diet, and  
27 their interaction affect a male's share of paternity. Early diet had no effect on paternity, but  
28 outbred males sired almost twice as many offspring as inbred males. We also found that  
29 males with a relatively long gonopodium (intromittent organ) had greater reproductive  
30 success. We demonstrate that inbreeding has important consequences because it negatively  
31 affects a key component of male fitness. Given there was no difference in adult mortality  
32 this finding can only be due to inbreeding negatively affecting sexually selected traits.

33

34 *Keywords:* heterozygosity, inbreeding depression, paternity, mosquitofish

35

36 **Introduction**

37

38 Evidence that inbreeding in animals negatively affects lifetime reproductive success or other  
39 close proxies of fitness, comes from two main streams of research: correlational studies  
40 (mainly of wild populations), and experimental studies on laboratory or captive populations  
41 that create inbred and outbred individuals. Correlational evidence for inbreeding depression  
42 includes: a) comparing traits among populations of the same species, specifically between  
43 small, isolated populations that have experienced varying degrees of inbreeding and large,  
44 outbred populations [1-4]; b) using molecular markers to obtain direct estimates of  
45 individual levels of inbreeding within a population and then relating these to fitness  
46 measures [i.e. heterozygosity-fitness correlations: HFCs; 5, 6-8]; c) calculating an individual's  
47 inbreeding coefficient from pedigree data and then relating this to a fitness measure [8-11].  
48 Although correlational studies often suggest that inbreeding lowers fitness, other factors  
49 cannot be ruled out. For example, inbred individuals might more often occur in peripheral  
50 environments that are of low quality such that there is a direct environmental effect on  
51 offspring phenotypes. More generally, the reduced fitness of inbred individuals might partly  
52 result from additive genetic effects rather than non-additive interactions within loci [i.e.  
53 lower heterozygosity; 12]. If focal traits are heritable, and individuals with lower values tend  
54 to mate with relatives because they have poorer dispersal ability or struggle to attract  
55 mates, this will lead to systematic overestimation of the negative effects of inbreeding [see  
56 the discussion in 12]. Inbred offspring will inherit lower trait values regardless of any direct  
57 effects of inbreeding. Studies that experimentally manipulate levels of inbreeding with  
58 controlled breeding designs offer a better approach when trying to quantify the costs of  
59 inbreeding.

60

61 To date, relatively few experimental studies have looked at the effects of inbreeding on  
62 estimates of fitness in non-domesticated animals. Of these, only a handful of studies have  
63 specifically looked at male fitness [e.g. 13, 14-16]. It therefore remains an open question as  
64 to the extent to which males are more susceptible than females to inbreeding depression.  
65 Mating success and fertilization success under sperm competition are major determinants of  
66 male fitness in most species [17-19]. Sexually selected traits that confer a mating or  
67 fertilization advantage are often under strong directional sexual selection and, in addition,  
68 they tend to be condition-dependent. Condition-dependence has been described as a form  
69 of 'genetic capture' because condition reflects how well the individual accumulates resources  
70 [20, 21]. This ability is likely to depend on many traits (e.g. foraging ability, food absorption  
71 efficiency, timing of development) all of which could be negatively affected by inbreeding. It is  
72 therefore plausible that due to sexual selection male mating success will show greater  
73 inbreeding depression than an equivalent naturally selected female trait such as fecundity.  
74 These data cannot, however, be obtained from studies that measure male lifetime  
75 reproduction output as they confound lifespan with reproductive success per potential  
76 breeding event (i.e. sexual selection).

77

78 Within experimental studies of animals that try to measure fitness there is high variation in  
79 the reported magnitude of inbreeding depression [e.g. 16, 22, 23, 24]. One possible source  
80 of variation is whether test individuals are exposed to a stressful environment [25, 26].  
81 Inbreeding might make individuals less effective at buffering themselves against  
82 environmental stress [27]. Dietary and temperature stress, for example, increase the extent  
83 of inbreeding depression in some species [28-31]. More generally, rearing animals in a  
84 benign lab environment (or plants in well-watered greenhouses) is often put forward to  
85 explain the absence of inbreeding depression in a laboratory study [32, 33]. Another  
86 potential source of variation in estimates of inbreeding depression might arise from the

87 evolutionary history of study populations affecting the baseline level and variability of  
88 homozygosity. For instance, as mean homozygosity in a population increases the difference  
89 in homozygosity between offspring of closely related individuals and those from random  
90 matings decreases [34]. This makes it harder to detect inbreeding depression. To date,  
91 studies that investigate how these potential sources of variation influence the effects of  
92 inbreeding on fitness-enhancing traits remain scant [but see 28, 34, 35].

93

94 Here we conduct an experiment to investigate how differences in inbreeding and juvenile  
95 diet (i.e. early stressful environment) influence a key component of male fitness, namely  
96 their reproductive success. We experimentally generated inbred and outbred male  
97 mosquitofish (*Gambusia holbrooki*) that were then reared on different diets as juveniles  
98 [36]. We then allowed males to compete freely for access to females and examined their  
99 share of paternity. The ability to gain paternity under sperm and mating competition is a key  
100 fitness component for males in species with high levels of female polyandry, such as *G.*  
101 *holbrooki*. Importantly our experimental design allows us to isolate sexual selection (as  
102 opposed to other forms of natural selection) as the cause of any inbreeding depression. In  
103 addition to our experimental manipulation of inbreeding using a controlled pedigree we  
104 measured each male's actual genome wide heterozygosity (based on >3000 SNPS) to shed  
105 light on how much variation in inbreeding is needed to detect inbreeding depression. We  
106 predict that under the competitive mating scenario we created that, if it occurred,  
107 inbreeding depression would be greater for males reared in a stressful environment.

108

## 109 **Methods**

110

111 *Origin of fish*

112

113 We used mosquitofish descended from wild caught fish collected in Canberra, Australia. The  
114 design used to create inbred and outbred males that were then reared on different diets is  
115 fully described in [36]. In brief, in each experimental block we mated individuals from two  
116 full sibling families (e.g. A and B in block 1, C and D in block 2 and so on). Brothers and sisters  
117 from full sibling families were paired to create inbred offspring (AA, BB;  $f=0.25$ ) and outbred  
118 offspring with reciprocal male-female crosses (AB, BA) to generate four cross-types. We set  
119 up 29 blocks (= maximum of 116 different family pairings types). The 452 male offspring  
120 from 192 broods (some experimental blocks had more than one pairing of a given type)  
121 were then reared individually in 1L tanks until maturity. Males then underwent a diet  
122 manipulation for 21 days between day 7 and day 28-post birth that lead to almost zero  
123 growth [36]. Fish on the control diet were fed *ad libitum* with *Artemia* nauplii twice a day  
124 (i.e. standard laboratory feeding regime) while fish on the restricted diet were fed 3mg of *A.*  
125 *nauplii* once every other day (i.e. < 25% of the control food intake). Broods were split evenly  
126 between the control and restricted diet treatment.

127

### 128 *Experimental design*

129

130 To determine whether inbreeding, diet, or their interaction predict paternity we set up  
131 mating trials in which four males, one per treatment, could compete and mate freely with a  
132 female in a 60L tank (n=31). Males were randomly assigned to each replicate and were not  
133 match for size (size range: 18.51 - 26.96 mm). Males were allowed to mate freely with a  
134 female for a week and then given a week to recover after the female was removed. The  
135 process was then repeated with two more females. The 93 test females were then placed in  
136 individual 1L tanks and allowed six weeks to give birth. They were checked for offspring  
137 twice daily. Once fish were removed from the treatment they were euthanized and  
138 preserved in absolute ethanol and stored at -20°C.

139

140 *Male morphology*

141

142 The phenotype of all males was measured prior to being placed in tanks with females. Males  
143 were anaesthetized by submersion in ice-cold water for a few seconds to reduce movement  
144 and then placed on polystyrene with a microscopic ruler (0.1 mm gradation) and  
145 photographed. We measured male standard length (SL = snout tip to base of caudal fin) and  
146 gonopodium length (intromittent organ modified from the anal fin) using Image J software  
147 [37]. The males were 28 – 37 weeks post-maturity and were marked with a small coloured  
148 dot for visual identification using fluorescent elastomer (Northwest Marine Technology, WA)  
149 injected subcutaneously behind the caudal fin. They were given at least four days recovery  
150 before going into 60L tanks to start mating trials. We calculated relative gonopodium size as  
151 the residuals from a linear regression of gonopodium size (log) on male standard length  
152 (log).

153

154 *Paternity analysis*

155

156 To determine male reproductive success and heterozygosity for the fish in our experiment  
157 we took tissue samples from each male (n=121), females that bred (n=79 of 93), and a  
158 maximum of 10 fry per female (n=628 offspring). Two of the 124 males (both outbred) were  
159 not found in the tank at the end of the trial (i.e. escaped or died) and therefore no tissue  
160 was available. For adults, DNA was extracted from the tail muscle/caudal fin. For fry DNA  
161 was extracted from the whole body, excluding the head. DNA was extracted using Qiagen  
162 DNeasy Blood & Tissue Kits following the manufacturer's instructions. After extraction, DNA  
163 samples were SNP genotyped. Full methods for the paternity analysis are in [38].

164

165 *Heterozygosity*

166

167 We estimated heterozygosity by using the number of markers that were scored as  
168 heterozygous divided by the total number of successfully classified markers for each fish.  
169 Based on over 3000 SNP loci we found that a brother-sister mating led to a significant  
170 decline in offspring heterozygosity ( $F_{(1,120)} = 215.1, P < 0.001$ ). The mean heterozygosity of  
171 inbred fish was 23.2% less than that of outbred fish (close to the expected 25% decline). The  
172 proportion of loci that were heterozygous was  $0.239 \pm 0.003$  in inbred males and  $0.311 \pm$   
173  $0.004$  in outbred males ( $n = 62, 59$ ).

174

175 *Statistical analysis*

176

177 We used Generalized Linear Mixed-effect models (GLMM) with Poisson error to test for  
178 fixed effects of inbreeding, relative heterozygosity (see below), diet, body size, relative  
179 gonopodium length, and the interaction between inbreeding and diet on how many  
180 offspring males sired. We used the *glmer* function in the *lme4* package in R 3.0.2 software  
181 [39]. To obtain a measure of relative heterozygosity we centered heterozygosity (mean = 0)  
182 within each inbreeding treatment. We could then test whether it explained additional  
183 variation in male reproductive success beyond that associated with the decline in absolute  
184 heterozygosity due to inbreeding. We also included the interaction between standardized  
185 heterozygosity and inbreeding to test for any difference in the effects of this additional  
186 variation in heterozygosity between inbred and outbred males (i.e. the effect will differ if  
187 there is a non-linear relationship between absolute heterozygosity and fitness). To account  
188 for overdispersion we included individual as a random effect [40]. We included tank as a  
189 random effect to account for potential non-independence. We included sire and dam as  
190 random effects. There was no effect so we present the simplified version of the model. All



191 model terms were tested for significance using the Anova function in the *car* package  
192 specifying Type III Wald chi-square tests. We removed non-significant interactions following  
193 [41]. All tests are two-tailed and alpha is set at 0.05.

194

## 195 **Results**

196

### 197 *Inbreeding*

198

199 On average, outbred males sired significantly more offspring than inbred males (Table 1, Fig.  
200 1). In 20 of 31 trials, the two outbred males sired more offspring than the two inbred males.  
201 More heterozygous males therefore had significantly greater reproductive success.

202

### 203 *Relative Heterozygosity*

204

205 We did not find any difference in how relative heterozygosity affected male reproductive  
206 success between inbred and outbred males (heterozygosity  $\times$  inbreeding,  $P = 0.350$ ). There  
207 was also no effect of relative heterozygosity on male reproductive success (Table 1).

208 Together these findings indicate that the standing variation in heterozygosity (i.e. that in  
209 outbred males) did not predict variation in male reproductive success.

210

### 211 *Diet*

212

213 We did not find an effect of diet on the number of offspring sired (Table 1). There was also  
214 no significant interaction between inbreeding status and diet ( $P = 0.586$ ).

215

### 216 *Male morphology*

217

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

220

## 221 **Discussion**

222

223 Inbreeding is expected to decrease fitness due to the negative effects of lower  
224 heterozygosity [42, 43]. Here we used a controlled breeding design combined with a  
225 genome wide SNP-based measure of heterozygosity to test whether inbreeding, as well as  
226 residual variation in heterozygosity, affects a key component of male fitness, namely male  
227 reproductive success. We found that one generation of inbreeding between full-siblings ( $f$   
228 =0.25) significantly lowered a male's ability to gain paternity by almost 50% (6.37 vs 3.76  
229 offspring). Outbred males sired significantly more offspring than inbred males when they  
230 had to compete for mates and fertilization opportunities. Sexual selection therefore favours  
231 outbred males. In addition, relative gonopodium length explained some of the remaining  
232 variation in reproductive success. Males with a longer gonopodium were significantly more  
233 successful. We found no evidence for an effect of diet or body size on male reproductive  
234 success. Nor did we find any effect of residual variation in heterozygosity once we  
235 accounted for the 23.2% decline in heterozygosity associated with inbreeding in our  
236 pedigree design (i.e. full-sibling parents versus unrelated parents).

237

### 238 *Heterozygosity and male fitness*

239 There is much indirect evidence from correlational studies that inbreeding reduces male  
240 reproductive success [8, 44-46]. In contrast, studies comparing the reproductive output of  
241 experimentally created inbred and outbred males have yielded less consistent results. For  
242 example, inbreeding depression had no effect on male offspring production in some

243 contexts in flour beetles [*Tribolium castaneum*; 14], while the proportion of offspring sired  
244 by inbred males was lower than that of outbred males in bulb mites (*Rhizoglyphus robini*)  
245 [*Rhizoglyphus robini*; 15]. In guppies (*Poecilia reticulata*), inbred males sired significantly  
246 fewer offspring than outbred males, but only when the inbreeding coefficient was at least  $f$   
247 =0.25 [i.e. two successive generations of full-sib breeding; 13]. Inbreeding is, in essence,  
248 simply a process that decreases heterozygosity. Our experiment therefore reveals a  
249 significant heterozygosity-fitness correlation (HFC) for male *G. holbrooki*. However, we also  
250 show that detecting this HFC could be difficult using standing variation in heterozygosity, as  
251 occurs in field studies [7, 47, 48]. Specifically, we found no effect of residual variation in  
252 heterozygosity for either inbred or outbred males. The latter males are roughly equivalent to  
253 the field population. It is therefore noteworthy that in a new study of field-caught males,  
254 albeit with a larger sample ( $n = 240$  putative sires), we detected a significant HFC for male  
255 reproductive success when males compete for females in semi-natural pools (Head et al.  
256 *submitted*—available for reviewers in supplementary material). One interpretation of this is  
257 that developing under more stressful field conditions exacerbates inbreeding depression.  
258  
259 Studies of inbreeding in wild populations usually fail to tease apart natural and sexual  
260 selection against inbred males. Reports of lower reproductive success for less heterozygous  
261 (i.e. inbred) males could be due to natural selection because of lower rates of survival [e.g.  
262 49, 50], which will, all else being equal, reduce their lifetime reproductive success; and/or  
263 because inbred males are less attractive to females or are weaker competitors [24, 51-53].  
264 In our experiment, we can eliminate natural selection through mortality as a source of  
265 variation in male reproductive success, so sexual selection most likely explains the lower  
266 reproductive success of inbred males. Interestingly, however, in another study we did not  
267 detect inbreeding depression for either sperm traits or male attractiveness in *G.*  
268 *holbrooki*, despite much larger sample sizes than in the current study (J. Marsh, R. Vega-

269 Trejo, M.L. Head, and M.D. Jennions 'unpublished data'). A lack of inbreeding depression in  
270 sperm traits in an introduced species (*G. holbrooki* are feral pests in Australia) could be  
271 attributed to low genetic variation due to founder effects [54]. However, the results we  
272 present in the current study highlight the need to look how inbreeding affects key fitness  
273 components, rather than phenotypic traits that are only indirect proxies for fitness. Given  
274 inbreeding depression for male reproductive success, future studies will need to look in  
275 more detail at the effects of inbreeding on attractiveness, ejaculate characteristics, male  
276 mating behaviour and fertilisation capacity.

277

#### 278 *Inbreeding depression in stressful and benign environments*

279 There is a trend for inbreeding depression to be higher in a more stressful environment [25,  
280 26]. By definition a more stressful environment is one that reduces fitness relative to a  
281 baseline environment [25]. Our low food diet resulted in almost zero growth over a three-  
282 week period [see 36], which strongly suggests that we created a stressful environment.  
283 Corroborating this, we have previously shown that this low food diet significantly reduces  
284 male attractiveness [55]. In studies of other taxa, mainly insects, a poor juvenile diet has  
285 been shown to reduce the ability of males to gain paternity [e.g. 56], which is mainly  
286 attributed to a lower sperm count and reduced sperm competitiveness [57, 58]. Elsewhere  
287 we have shown that, controlling for age, a poor juvenile diet reduces sperm reserves and  
288 sperm replenishment rates in younger male *G. holbrooki* (see Vega-Trejo et al *submitted*—  
289 available for reviewers in supplementary material). The males in our current experiment  
290 were, however, sufficiently old (28- 37 weeks post-maturation) that those on both diets  
291 should have had similar sperm production rates so the juvenile diet was not stressful for  
292 sperm production. If sperm numbers are a major determinant of male reproductive success  
293 this would partly explain why there was no main or interactive effect of diet on male

294 reproductive success. Again, however, this then raises the question of the proximate  
295 mechanism causing inbred males to have significantly lower paternity.  
296  
297 Studies of a range of taxa report a weak or no relationship between inbreeding depression  
298 and the level of dietary stress [effect size  $r = -0.13$  to  $0.02$ ; 59, 60, 61], but most of the focal  
299 traits measured in these studies are naturally selected. Sexually selected traits that affect  
300 male reproductive success are predicted to be more sensitive to inbreeding depression  
301 because of their stronger links with fitness [21, 62-64], and more sensitive to environmental  
302 stress because they tend to be condition-dependent [65, 66]. It is therefore intriguing that  
303 we found significant inbreeding depression for male reproductive success but no effect of  
304 diet on a male's share of paternity. More generally, additional studies of many more taxa are  
305 needed to establish whether sexually selected traits show the same pattern as naturally  
306 selected traits [25, 26] with respect to whether a more stressful environment elevates  
307 inbreeding depression.

308

### 309 *Morphological predictors of male fitness*

310

311 Males with a relatively long gonopodium for their body size had significantly higher  
312 reproductive success, even taking into account the effects of heterozygosity. This  
313 corroborates results from another study of *G. holbrooki* in semi-natural pools (Head et al.  
314 *submitted*—available for reviewers in supplementary material). Several studies of poeciliid  
315 fishes report a link between relative gonopodium length and male fitness [67-71] but see  
316 Booksmythe et al. 2016). Male body size is another trait that is often implicated in sexual  
317 selection in *G. holbrooki* but in the current study we found that it had no effect on male  
318 reproductive success. Male mosquitofish use a coercive mating tactic in which they position  
319 themselves behind the female and then thrust their gonopodium forward in an attempt to

320 transfer sperm into the female's gonoduct [72, 73]. Male size is highly variable and small  
321 males have greater manoeuvrability that seems to increase their propensity to sneak  
322 copulations [74]. Large males are, however, socially dominant, and might additionally  
323 transfer more sperm per encounter because they have larger sperm reserves [75]. This could  
324 compensate for the reduced ability of larger males to obtain sneak copulations [74-76]. The  
325 net relationship between male body size and reproductive success is likely to depend on the  
326 social context, including the absolute size difference between a male and female and the  
327 extent of male-male competition for matings [74]. In another paternity study we found that  
328 smaller males had significant greater reproductive success when they competed freely for  
329 mates in 24 semi-natural pools that varied in the adult sex ratio and habitat complexity  
330 (Head et al. *submitted*—available for reviewers in supplementary material). Spatio-temporal  
331 variation in how male size affects reproductive success seems likely given the wide size  
332 range at which males reach sexual maturity (there is almost no post-maturation growth),  
333 even when they are reared under identical laboratory conditions.

334

### 335 *Conclusions*

336

337 We conducted an experiment that showed that inbreeding reduces a key fitness component  
338 (share of paternity) of male *Gambusia holbrooki*. Our design removed most sources of  
339 natural selection (e.g. offspring and adult survival, time to maturation), so the lower success  
340 of inbred males strongly suggests that inbreeding affects sexual selected traits. This is  
341 important as sexual selection against inbred males could reduce the genetic load [33]. If  
342 inbred males are less likely to mate and/or fertilize eggs, this will reduce the frequency of  
343 deleterious recessive alleles and potentially lower the risk of extinction in small populations  
344 [77-80]. This possibility, if generally true in other taxa, could be profitably incorporated into  
345 models of population viability.

346

347 Our study is also a reminder that standing variation in heterozygosity plays an important role  
348 in the likelihood of detecting inbreeding depression, which might explain variation in  
349 reported level of inbreeding depression in other studies [e.g. 7, 47, 48]. Standing variation in  
350 heterozygosity, hence the use of heterozygosity-fitness correlations, was insufficient to  
351 detect inbreeding depression in our study as there was no effect of relative heterozygosity  
352 on paternity. We only detected inbreeding depression between our inbreeding treatment  
353 led to a 23% decline in heterozygosity. Given the potential for inbreeding to shape the  
354 evolution of key life history traits [81], more studies are needed that quantify inbreeding  
355 depression by taking an experimental approach and then measure fitness as directly as  
356 possible (i.e. reproductive success not simply phenotypic traits).

357

#### 358 **Competing interests**

359 The authors declare that they have no competing interests.

360

#### 361 **Authors' contributions**

362 R.V.T., M.L.H., and M.D.J. designed the study. R.V.T carried out the experimental work. J.S.K.  
363 analysed the paternity data. R.V.T., M.L.H., and M.D.J. analysed the data and wrote the  
364 manuscript. All the authors contributed substantially to revisions, and gave final approval for  
365 publication.

366

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376



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Table 1. Results from the mixed model with parameter estimates and chi square ( $\chi^2$ ) tests for heterozygosity, inbreeding, food treatment, size, and relative gonopodium size. P-values in bold indicate significant values.

	Predictor	Estimate	SE	$\chi^2$	P
Number of offspring	Intercept	-17.295	13.888	1.551	0.213
	Relative heterozygosity	0.114	0.201	0.319	0.572
	Inbreeding (inbred)	-0.943	0.399	5.596	<b>0.018</b>
	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 [ln(mm)]	0.483	0.212	5.179	<b>0.023</b>

Figure 1. Mean number of offspring ( $\pm$  SE) sired by outbred and inbred males.

