

Experimental evidence for sexual selection against inbred males

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Summary

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

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

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

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

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

Key-words: heterozygosity, inbreeding depression, mosquitofish, paternity, reproductive success

Introduction

Environments that are spatially fragmented result in small, isolated populations in which relatives are more likely to mate with each other (Lande 1988; Keller & Waller 2002; Becker *et al.* 2016). Mating between relatives often decreases genome-wide heterozygosity in the resultant offspring, which can reduce the mean phenotypic value of traits putatively associated with fitness, so-called 'inbreeding depression' (Falconer & Mackay 1996; Lynch & Walsh 1998). Inbred individuals are assumed to be less fit due to greater expression of deleterious, recessive alleles (dominance hypothesis) and/or due to homozygosity at loci

where heterozygosity confers an advantage (overdominance) (Charlesworth & Charlesworth 1987, 1999). Traits that are closely related to fitness are predicted to be more likely to show inbreeding depression (DeRose & Roff 1999), because strong directional selection promotes fixation of advantageous alleles, and rapidly eliminates deleterious dominant alleles (Lynch & Walsh 1998; DeRose & Roff 1999). By measuring traits that are only weakly related to fitness researchers underestimate the true effects of inbreeding on fitness. More studies are needed that directly quantify the effects of inbreeding on fitness or, given the logistic challenges of measuring net fitness, studies that focus on key fitness components (Hedrick & Kalinowski 2000; Reed & Frankham 2003; Huisman *et al.* 2016).

To date, relatively few experimental studies have looked at the effects of inbreeding on fitness estimates in

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non-domesticated animals. Of these studies, most focus on female reproductive output, or non-sex-specific life-history traits (e.g. Pilakouta & Smiseth 2016), and only a handful have specifically looked at male fitness. For example Zajitschek *et al.* (2009) showed that highly inbred males sire fewer offspring than outbred males; Michalczyk *et al.* (2010) reported that inbreeding depression reduces sperm competitiveness, which can affect male's fertilisation; Konior, Keller & Radwan (2005) estimated the proportion of offspring sired by outbred and inbred males and found that it was lower for outbred males; and Bickley *et al.* (2013) showed a tendency for inbred males to sire fewer offspring when in direct competition with outbred males.

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

There is high variation in the reported magnitude of inbreeding depression in the available experimental studies of wild animals that try to measure fitness (e.g. Meagher, Penn & Potts 2000; Harano 2011; Bickley *et al.* 2013; Thonhauser, Raveh & Penn 2014). One possible source of variation is whether or not test individuals experience a stressful environment (Armbruster & Reed 2005; Fox & Reed 2011). Inbreeding might result in individuals less able to buffer their development against environmental stress (Miller 1994). Dietary and temperature stress, for example can increase levels of inbreeding depression (e.g. Dahlggaard & Loeschcke 1997; Kristensen *et al.* 2008; Auld & Henkel 2014; Freitag *et al.* 2014) as can stress arising from intraspecific competition (Meagher, Penn & Potts 2000; Joron & Brakefield 2003; Yun & Agrawal 2014). More generally, rearing animals in a benign laboratory environment (or plants in greenhouses) is often

invoked to explain the absence of inbreeding depression in laboratory studies (Duarte *et al.* 2003; Enders & Nunney 2012). Another potential source of variation in estimates of inbreeding depression might be that the evolutionary history of study populations affects the baseline level of heterozygosity. For instance as mean heterozygosity in a population decreases the difference in heterozygosity between offspring of closely related individuals and those from random matings decreases (Pekkala *et al.* 2014). This makes it harder to detect inbreeding depression (see also Miller & Coltman 2014). To date, experimental studies that investigate how these different potential sources of variation influence the effects of inbreeding on fitness-enhancing traits remain scant (but see Dahlggaard & Loeschcke 1997; Reed & Frankham 2003; Pekkala *et al.* 2014).

Here we conduct an experiment to investigate how differences in inbreeding level and juvenile diet (manipulated to create a stressful environment) influence a key component of male fitness, namely reproductive success when competing for mates and fertilisation opportunities in the mosquitofish, *Gambusia holbrooki*. *Gambusia holbrooki* is a poeciliid fish endemic to North America, but now introduced world-wide. Mosquitofish are non-migratory, and are often resident in relatively small bodies of water, such as ponds and streams (Pyke 2005). This makes it likely that inbreeding occurs naturally if a few fish become isolated in a small area. There is sufficient genetic variation in our study population for inbreeding to lead to a detectable, and predicted, decline in heterozygosity (see Results). Mosquitofish have internal fertilisation and males transfer sperm to females via a modified anal fin called the gonopodium (Pyke 2005). Males do not court, but perform coercive 'sneak' copulations in which they approach a female from behind and thrust their gonopodium towards her gonopore (Bisazza & Marin 1995; Langerhans 2011). Male size is highly variable and small males have greater manoeuvrability that seems to increase their propensity to sneak copulations (Pilastro, Giacomello & Bisazza 1997). Large males are, however, socially dominant and might transfer more sperm per encounter because they have larger sperm reserves (O'Dea, Jennions & Head 2014). Female size varies considerably and is strongly correlated with fecundity (Bisazza, Marconato & Marin 1989; Callander, Backwell & Jennions 2012). Females give birth to live young. Finally, standing variation in heterozygosity is positively correlated with male reproductive success when males compete for mates in experimental ponds (Head *et al.* 2016).

We experimentally generated inbred and outbred males that were initially reared on different diets as juveniles (Vega-Trejo, Head & Jennions 2016). We then allowed adult males to compete freely for access to females and quantified their share of paternity. The ability to gain paternity under sperm and mating competition is a key male fitness component in species with high levels of female polyandry, such as *G. holbrooki* (Pilastro,

Giacomello & Bisazza 1997; Bisazza, Vaccari & Pilastro 2001). Importantly, our experimental design allows us to isolate sexual selection (as opposed to other forms of natural selection) as the mechanism driving any inbreeding depression because we eliminated variation in male mortality. In a second experiment we tested, and confirmed, that being inbred did not affect a male's non-competitive fertilisation ability and/or elevate embryo mortality. We established this by artificially inseminating females with either an inbred or an outbred male's sperm and noting their realised fecundity (i.e. offspring at birth). In addition to the experimental manipulation of inbreeding status using a controlled pedigree we directly estimated each male's genome wide heterozygosity (based on >3000 SNPs) to estimate whether the direct use of an actual estimate of heterozygosity provides a more powerful means to detect inbreeding depression than the binary division of males into inbred and outbred. Our design also allowed us to test the prediction that inbreeding depression for reproductive success would be greater for males reared in a stressful juvenile environment.

Materials and methods

ORIGIN AND MAINTENANCE OF FISH

We used mosquitofish descended from wild caught fish collected in Canberra, Australia. The design that we used to create inbred and outbred males that were then reared on different diets, is fully described in Vega-Trejo, Head & Jennions (2016). In brief, in each experimental block we mated individuals from two full sibling families (e.g. A and B in block 1, C and D in block 2, etc). Brothers and sisters from full sibling families were paired to create inbred offspring (AA, BB; $f = 0.25$) and outbred offspring with reciprocal male-female crosses (AB, BA) to generate four cross-types. We set up 29 blocks (= maximum of 116 different family pairings types). The 452 male offspring from 192 broods (some experimental blocks had more than one pairing of a given type) were then reared individually in 1 L tanks that were distributed randomly throughout a temperature-controlled room (14 : 10 h photoperiod at 28 °C). Males underwent a diet manipulation for 21 days from day 7 to 28 post birth that lead to almost zero growth (Vega-Trejo, Head & Jennions 2016). Fish on the control diet were fed *ad libitum* with *Artemia* nauplii twice daily (i.e. standard laboratory feeding regime), whereas fish on the restricted diet were fed 3 mg of *Artemia* nauplii once every other day (i.e. <25% of the control diet). Broods were split evenly between the control and restricted diet.

EXPERIMENTAL DESIGN — COMPETITIVE MATING SCENARIO

To determine whether inbreeding, diet or their interaction predict paternity we set up mating trials in which four unrelated males, one per treatment, could compete and mate freely with a stock virgin female in a 60 L tank ($n = 31$). Males were randomly assigned to each replicate and were not matched for size (size range: 18.51–26.96 mm). We have previously shown that inbred and outbred males do not differ in size at maturity (Vega-Trejo,

Head & Jennions 2016). After a week we removed the female and gave the males a week to recover. The process was then repeated with two more females. The four males in each replicate were kept together for all three trials. The 93 test females were each placed in individual 1 L tanks, and we checked twice daily for 6 weeks whether she had given birth. Offspring were collected immediately and preserved (see below). Adults were killed, preserved in absolute ethanol and stored at -20 °C.

MALE MORPHOLOGY

All males were measured before we placed them in tanks with females. Males exhibit minimal growth after maturation (Cabral & Marques 1999; Pyke 2005; Kahn, Livingston & Jennions 2012), so we did not remeasure them between trials. Males were anaesthetised by submersion in ice-cold water for a few seconds to reduce movement, placed on polystyrene with a microscopic ruler (0.1 mm gradation), and photographed. We measured male standard length (SL = snout tip to base of caudal fin) and gonopodium length (intromittent organ modified from the anal fin) using Image J software (Abramoff, Magelhaes & Ram 2004). The test males were 28–37 weeks post-maturity and were marked with a small coloured dot for visual identification using fluorescent elastomer (Northwest Marine Technology, Shaw Island, WA, USA) injected subcutaneously behind the caudal fin. They had at least 4 days to recover before being placed in 60 L tanks to start mating trials. We calculated relative gonopodium size as the residuals from a linear regression of gonopodium size (log) on SL (log) (Bookmythe *et al.* 2016).

PATERNITY ANALYSIS

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

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

HETEROZYGOSITY

We estimated heterozygosity (H) as the number of SNP loci that were scored as heterozygous divided by the total number of successfully classified loci (L) for each male who was a potential sire in the competitive mating experiments (F_{het}). This is essentially a measure of genome wide heterozygosity. F_{het} is identical to $1 - F_{\text{hom}}$ in Bérénos *et al.* (2016); and to H/L in Szulkin, Bierne & David (2010, table 2), albeit that there are minor differences in L among individual males; $L = 3360 \pm 2.68$ (mean \pm SE) loci per male were successfully classified. We found that a brother-sister mating led to a significant decline in offspring F_{het} ($F_{1,120} = 215.1$, $P < 0.001$) because the proportion of classified

loci per male that were heterozygous was 0.239 ± 0.025 (mean \pm SD; range: 0.185–0.288) in inbred males ($n = 62$) and 0.311 ± 0.028 (mean \pm SD; range: 0.263–0.378) in outbred males ($n = 60$). The mean heterozygosity of inbred fish was therefore 23.2% less than that of outbred fish, close to the expected 25% decline in F_{het} . We also calculated the mean heterozygosity of the 79 females that bred and of the 628 offspring that were genotyped. For the females, F_{het} was 0.314 ± 0.003 ; and for the offspring, F_{het} was 0.318 ± 0.001 . These values do not differ significantly from that for outbred males (one-way ANOVA: $F_{2,763} = 2.576$, $P = 0.077$, $n = 60$ males, 79 females, 628 offspring). There is therefore no detectable sex difference in heterozygosity, and no decline in heterozygosity in the mating trial between outbred individuals in the parental and offspring generation.

EXPERIMENTAL DESIGN – NON-COMPETITIVE MATING SCENARIO

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

STATISTICAL ANALYSIS

We used generalised linear mixed-effect models (GLMM) with Poisson error to test for fixed effects of inbreeding, diet, body size, relative gonopodium length and the interaction between inbreeding and diet on how many offspring each male sired. There is no significant effect of inbreeding on relative gonopodium length (GLMM: $\chi^2 = 0.529$; $P = 0.467$; $n = 124$). Consequently, including relative gonopodium length in the model does not mask any effects of inbreeding that might act via an effect on gonopodium length (i.e. it is not a covariate measured post-treatment *sensu* Gelman & Hill 2007, p. 188). We used the *glmer*

function in the *lme4* package in *R* 3.0.2 software (R Development Core Team, 2012). As already noted, Heterozygosity (F_{het}) differed greatly between inbred and outbred males. Our main test for whether heterozygosity affects male success under mating competition is therefore simply the effect of inbreeding status. However, to test whether heterozygosity, after controlling for that associated with inbreeding status, explained additional variation in paternity success, we also standardised heterozygosity. We centred F_{het} so that the mean was 0 for each inbreeding treatment (hereafter F^*_{het}). We then ran the final model including F^*_{het} and its interaction with inbreeding status. An interaction would arise if there is a nonlinear relationship between F_{het} and paternity success. To account for overdispersion we included individual as a random effect (Harrison 2014). Following this correction our data was underdispersed (dispersion parameter = 0.33) and conservative. We included mating trial tank as a random effect to account for potential non-independence. We also included sire and dam identity as random effects in the final model, even though they explained almost no variation in male reproductive success. This can partly be attributed to low statistical power to detect additive genetic variation underlying male reproductive success as, for example of the 60 sires that provided sons we used in the competitive mating trials, the mean number of sons per sire was 2.07 (range 1–6). All fixed model terms were tested for significance using the *Anova* function in the *car* package specifying Type III Wald chi-square tests. We removed non-significant interactions following Crawley (2005). All tests are two-tailed and alpha is set at 0.05.

To test whether females that were artificially inseminated by inbred males produced fewer broods than those inseminated with sperm from outbred males we used a GLMM with Binomial error. Whether or not a female produced a brood (i.e. 0, 1) was the response variable. Inbreeding status, diet and their interaction were included as fixed factors. We included male identity as a random effect to correct for repeated measurements. We also tested whether male inbreeding status influenced how many fry a female gave birth to. To do so, we used the mean number of fry produced by females (excluding those that did not breed) for each male as the response variable in a GLM with a quasipoisson error structure to account for overdispersion. Male inbreeding status, diet and their interaction were included as fixed factors. We again removed non-significant interactions following Crawley (2005).

To estimate the standardised difference among means we calculated Hedges' g following Rosenberg, Rothstein & Gurevitch (2013). By convention we refer to $r = 0.1$, 0.3 and 0.5 as small, medium, and large effect sizes respectively (Cohen 1988).

Results

MALE REPRODUCTIVE SUCCESS UNDER A COMPETITIVE MATING SCENARIO

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

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

	Predictor	Estimate	SE	χ^2	<i>P</i>
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	0			
	tank				

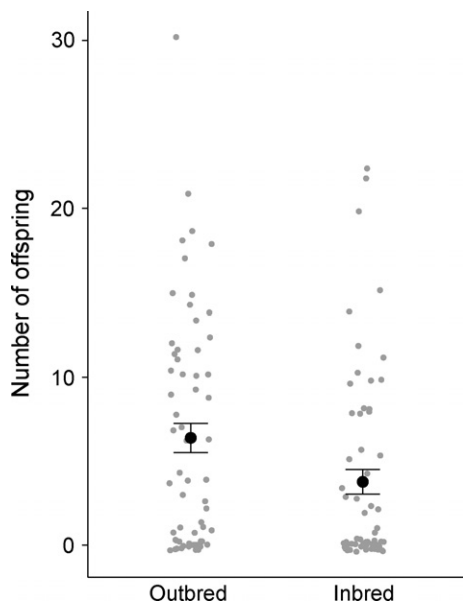


Fig. 1. Mean number of offspring (\pm SE) sired by outbred and inbred males ($n = 122$ males genotyped; 60 outbred and 62 inbred). Raw data are represented by dots.

HETEROZYGOSITY CONTROLLING FOR INBREEDING STATUS

We did not find any significant difference in how F^*_{het} affected male reproductive success between inbred and outbred males ($F_{het} \times$ inbreeding, $\chi^2 = 0.873$; $P = 0.350$). There was also no significant effect of F^*_{het} on male reproductive success (Table 1). Together these findings indicate that the residual variation in heterozygosity (i.e. F^*_{het} in outbred males) did not predict variation in male

reproductive success. We also tested whether a GLMM using F_{het} was a better predictor of male reproductive success than a GLMM using inbreeding status (the other fixed model terms: diet, body size, relative gonopodium length and an interaction between diet and inbreeding status or F_{het}). The amount of variation explained was identical ($R^2 = 0.117$), which confirms that in the analysis using inbreeding status and F^*_{het} , the extra information from the use of actual heterozygosity estimates did not allow us to explain significantly more variation than obtained based solely on the difference in heterozygosity generated by the creation of inbred and outbred males.

DIET

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

MALE MORPHOLOGY

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

MALE REPRODUCTIVE SUCCESS UNDER A NON-COMPETITIVE MATING SCENARIO

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

Discussion

Inbreeding is expected to lower fitness due to the negative effects of decreased heterozygosity (Charlesworth & Charlesworth 1987; Lynch & Walsh 1998). Here we used a controlled breeding design combined with a genome wide SNP-based measure of heterozygosity to test whether inbreeding, as well as residual variation in heterozygosity, affects a key component of male fitness, namely reproductive success when males compete for fertilisation opportunities. We found that one generation of inbreeding

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

	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			

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

	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

between full-siblings ($f = 0.25$), leading to a 23.2% decline in the proportion of SNP loci that were heterozygous, significantly decreased paternity success (6.37 vs. 3.76 offspring per male).

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

HETEROZYGOSITY AND MALE FITNESS

There is indirect evidence from correlational field studies that inbreeding reduces male reproductive success

(Chapman & Sheldon 2011; Cain *et al.* 2014; Frère, Chandrasoma & Whiting 2015; Huisman *et al.* 2016). In contrast, studies comparing the reproductive output of experimentally created inbred and outbred males have yielded less consistent results. For example inbreeding depression had no effect on offspring production under a non-competitive scenario in male wild house mice and male flour beetles (Meagher, Penn & Potts 2000; Michalczyk *et al.* 2010), whereas the proportion of offspring sired by inbred males was lower than that of outbred males in bulb mites (*Rhizoglyphus robini*; Konior, Keller & Radwan 2005). In guppies (*Poecilia reticulata*), inbred males sired significantly fewer offspring than outbred males, but only when the inbreeding coefficient was at least $f = 0.375$ (i.e. two successive generations of full-sib breeding; Zajitschek *et al.* 2009). Inbreeding is, in essence, simply a process that decreases heterozygosity, which is why heterozygosity is used as a proxy for inbreeding (Miller & Coltman 2014; Béréanos *et al.* 2016). Our experiment reveals a significant heterozygosity-fitness correlation (HFC) for male *G. holbrooki*. However, we also show that detecting this HFC could be difficult using standing variation in heterozygosity, as occurs in field studies (Coltman & Slate 2003; Chapman *et al.* 2009; Szulkin, Bierne & David 2010). Specifically, we found no effect of residual heterozygosity (F^*_{het}) on reproductive success for either inbred or outbred males. The variance in (residual) heterozygosity of outbred males in our study should be equivalent to that of males in the field population. [The only caveat is that the variance in heterozygosity in males in the field will be greater if there is inbreeding in the wild. The extent of any difference in heterozygosity will increase with the natural rate of occurrence of inbreeding. We specifically eliminated any such inbreeding in our study by always pairing unrelated fish to create outbred males (Miller & Coltman 2014; Szulkin, Bierne & David 2010)]. It is therefore intriguing that in a new study of field-caught males, albeit with a larger sample ($n = 240$ putative sires), we detected a significant HFC for male reproductive success when males competed for females in 24 semi-natural pools (M.L. Head, A. Kahn, J.S. Keogh & M.D. Jennions, unpublished data). One interpretation of this difference in the reported effect of heterozygosity is that when males develop under natural field conditions this exacerbates inbreeding depression (see Throver & Hard 2009). Another possibility is that there is actually considerable variation in the relatedness of mates in the field, which elevates variation in heterozygosity. This source of variation was eliminated in our study due to the controlled breeding design. That is F^*_{het} is heterozygosity after removing effects of parents mating with close relatives.

Studies of inbreeding in the wild generally fail to tease apart natural and sexual selection against inbred males. Reports of lower reproductive success for less heterozygous (i.e. inbred) males could be due to natural selection because of lower rates of survival (e.g. Frommen *et al.*

2008; Mulard *et al.* 2009; Velando, Barros & Moran 2015), which will, all else being equal, reduce their lifetime reproductive success; and/or because inbred males are less attractive to females (including discrimination at the gametic level; Crean & Bonduriansky 2014) or are weaker mating or sperm competitors (Aspi 2000; Meagher, Penn & Potts 2000; Joron & Brakefield 2003; Okada *et al.* 2011). However, sperm traits may not always be affected by inbreeding depression (Mehlis *et al.* 2012; Opatová *et al.* 2016). In our experiment, we can eliminate natural selection through mortality as a major source of variation in male reproductive success (the two male deaths reduce our estimate of inbreeding depression). We can also rule out an effect of male inbreeding status on embryo mortality. When we artificially inseminate virgin females using the sperm of a single male, inbred and outbred males produced the same number of offspring. This finding is similar to studies that have found that the effects of inbreeding depression are not evident under a non-competitive mating scenario (e.g. Meagher, Penn & Potts 2000; Michalczyk *et al.* 2010). Sexual selection is therefore the most likely explanation for the lower reproductive success of inbred males. Indeed, by definition, it is the only explanation (aside from Type I error) if sexual selection is broadly defined as variation in reproductive success arising from competition for gametes. It should be noted, however, that competitive interactions in the wild might lead to natural selection on traits that indirectly affect the expression of sexually selected traits (e.g. due to trade-offs in investment) and thereby amplify inbreeding depression on traits under sexual selection.

An obvious question to ask is: what traits account for sexual selection against inbred male *G. holbrooki*? Interestingly, in another study we did not detect inbreeding depression in *G. holbrooki* for sperm traits (velocity and sperm count) or for male attractiveness (based on two-choice association tests), despite much larger sample sizes than in this study (J. Marsh, R. Vega-Trejo, M.D. Jennions & M.L. Head, unpublished data; data and analysis available on request). The lack of inbreeding depression in sperm traits could be attributed to low genetic variation due to founder effects (Ayres, Pettigrove & Hoffmann 2010) because *G. holbrooki* are an introduced feral pest species in Australia. Low genetic variation reduces the magnitude of the difference in heterozygosity between inbred and outbred males. However, the inbreeding depression we report here for actual reproductive success makes this a weak explanation. Ultimately, the results we present here highlight the need to look at how inbreeding affects key fitness components, and not only look at phenotypic traits (such as sperm count) that are only indirect proxies for fitness. Based solely on sperm velocity and sperm count, we would not predict a decline in the fertilisation ability of inbred males. Of course, inbred males might not have less competitive ejaculates. They might simply be less successful at initially inseminating females. In a separate study we used artificial

insemination, controlling for sperm number, to test whether inbred males have less competitive ejaculates than outbred males (J. Marsh, R. Vega-Trejo, M.D. Jennions & M.L. Head, unpublished data). There is evidence that the greater the difference in heterozygosity between two competing males the higher the share of paternity gained by the more heterozygous male, suggesting that inbred males will, on average, have less competitive ejaculates.

INBREEDING DEPRESSION IN STRESSFUL AND BENIGN ENVIRONMENTS

Inbreeding depression tends to be higher in a more stressful environment (Armbruster & Reed 2005; Fox & Reed 2011). By definition a more stressful environment is one that reduces fitness relative to a baseline environment (Armbruster & Reed 2005). Our low food diet resulted in almost zero growth over a 3-week period (see Vega-Trejo, Head & Jennions 2016), which strongly suggests that we created a stressful environment. Corroborating this, we have previously shown that this diet significantly reduces male attractiveness measured as female association time (Kahn, Livingston & Jennions 2012). It should, however, be noted that in this study a low food diet did not reduce a male's ability to gain paternity when competing for mates. Studies of other taxa, mainly insects, show that a poor juvenile diet can reduce the ability of males to gain paternity (e.g. Moreau *et al.* 2007). This is mainly attributed to a lower sperm count and reduced sperm competitiveness (Rahman, Kelley & Evans 2013; Muller *et al.* 2015). Elsewhere we have shown that, controlling for age, a poor juvenile diet reduces sperm reserves and sperm replenishment rates in younger male *G. holbrooki* (Vega-Trejo, Jennions & Head 2016). The males in our current experiment were, however, sufficiently old (28–37 weeks post-maturation) that juvenile diets should not have affected sperm production. If sperm number is a major determinant of male reproductive success this would partly explain why there was no main or interactive effect of diet on male success. Again, however, this raises the question of the proximate mechanism causing inbred males to have lower paternity.

Studies of a range of taxa report a weak or no relationship between inbreeding depression and the level of dietary stress (effect size $r = -0.13$ to 0.02 ; Fox *et al.* 2011; Reed & Bryant 2001; Reed *et al.* 2003), but most of the focal traits measured in the primary studies are naturally selected. Sexually selected traits that affect male reproductive success are predicted to be more sensitive to inbreeding depression because of their tight links with fitness (Tomkins *et al.* 2004; Drayton *et al.* 2007; Bolund *et al.* 2010; Mallet & Chippindale 2011), and their greater sensitivity to environmental stress because they tend to be condition-dependent (David *et al.* 2000; Ingleby, Hunt & Hosken 2010). It is therefore intriguing that we found significant inbreeding depression for male reproductive success, but no effect of diet. It is possible that we did not

find a dietary effect because the stressful environment was simply not stressful enough or because it was only experienced early in life. More generally, we suggest that studies of many more taxa are needed to establish whether sexually selected traits show the same pattern as naturally selected traits (Armbruster & Reed 2005; Fox & Reed 2011) with respect to whether a more stressful environment elevates inbreeding depression.

MORPHOLOGICAL PREDICTORS OF MALE FITNESS

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

Conclusions

We conducted an experiment that showed that inbreeding reduces a key fitness component (share of paternity) of male *G. holbrooki*. Our design removed most sources of natural selection (e.g. offspring and adult survival), and our artificial insemination experiment revealed no effect of male inbreeding on embryo mortality, so the lower reproductive success of inbred males strongly suggests that inbreeding affects sexually selected traits. This is important as sexual selection against inbred males could reduce the genetic load (Enders & Nunney 2012). If inbred males are less likely to mate and/or fertilise eggs, this will reduce the frequency of deleterious recessive alleles and could potentially lower the risk of extinction in small populations (Whitlock 2000; Radwan *et al.* 2004; Sharp & Agrawal 2008; Hollis, Fierst & Houle 2009). This possibility, if generally true in other taxa, could be profitably incorporated into models of population viability, as inbreeding can shape the evolution of key life-history traits (Charpentier, Widdig & Alberts 2007). Of course, we readily acknowledge that our estimate of the effect of inbreeding on males is based on reproductive success in a specific context (four males competing for a female). This

is not an unnatural situation given the wide range in adult sex ratios seen in the field (e.g. Cameron 2004; Donald 2007), but the strength of sexual selection might change when there is a less male-biased sex ratio (but see Henshaw, Kahn & Fritzsche 2016).

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

Authors' contributions

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

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Data accessibility

All data associated with this study have been deposited in the Dryad Digital Repository <http://dx.doi.org/10.5061/dryad.6d87p> (Vega-Trejo *et al.* 2016).

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Supporting Information

Details of electronic Supporting Information are provided below.

Appendix S1. Heterozygosity based on SNPs.