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Why does inbreeding reduce male paternity? Effects on sexually selected traits

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Mating with relatives has often been shown to negatively affect offspring fitness (inbreeding depression). There is considerable evidence for inbreeding depression due to effects on naturally selected traits, particularly those expressed early in life, but there is less evidence of it for sexually selected traits. This is surprising because sexually selected traits are expected to exhibit strong inbreeding depression. Here, we experimentally created inbred and outbred male mosquitofish (*Gambusia holbrooki*). Inbred males were the offspring of matings between full siblings. We then investigated how inbreeding influenced a number of sexually selected male traits, specifically: attractiveness, sperm number and velocity, as well as sperm competitiveness based on a male's share of paternity. We found no inbreeding depression for male attractiveness or sperm traits. There was, however, evidence that lower heterozygosity decreased paternity due to reduced sperm competitiveness. Our results add to the growing evidence that competitive interactions exacerbate the negative effects of the increased homozygosity that arises when there is inbreeding.

KEY WORDS: **Inbreeding depression, mate choice, paternity, sexual selection, sperm competition, poeciliid.**

Studies of wild animals often support the widespread expectation that being inbred reduces an individual's fitness (i.e., inbreeding depression) (reviewed in Hedrick and Kalinowski 2000; Keller and Waller 2002; Chapman et al. 2009). However, the contribution of sexually selected traits to lowering fitness is often unclear: relatively few studies have both experimentally manipulated the inbreeding status of males and ruled out potential confounding effects of natural selection on their reproductive success (but see, e.g., Drayton et al. 2010; Bolund et al. 2010; Zajitschek and Brooks 2010; Valtonen et al. 2014). At the within-population level, the comparative importance of sexual and natural selection in reducing the relative fitness of inbred and outbred males is unclear. There is, in addition, a potential link between lower relative fitness of inbred males and a population-level effect, but identifying this could depend on the causes of their reduced fitness. For example, sexual selection could exacerbate the detrimental effects of inbreeding in small populations. If inbred males are less likely to mate, even if they survive to adulthood, then the effective population size is smaller than that predicted based solely on survival to adulthood (Lynch and Walsh 1998).

There is a longstanding argument that male sexually selected traits will exhibit intense inbreeding depression (Brown 1997). This is partly because these traits, as with other major life-history traits, are important determinants of fitness, with a history of strong selection. Such traits are predicted to be more adversely affected by the bearer's inbreeding status because strong selection reduces the frequency of deleterious alleles that are dominant, and most deleterious alleles are therefore expected to be recessive (DeRose and Roff 1999; Roff and Emerson 2006). The resultant directional dominance means that alleles that lead to a beneficial increase (or decrease) in a trait are on average dominant over those that reduce (or increase) the trait (Wolak and Keller 2014). Inbreeding exposes these recessive mutations by increasing homozygosity. Consequently, sexually selected traits should show greater inbreeding depression than traits, such as minor morphological features that are weaker determinants of fitness so that directional dominance is weaker or absent (Lynch and Walsh 1998, p270; Cotton et al. 2004). Furthermore, sexually selected traits are often condition-dependent, and inbreeding status can adversely affect condition through its effects on the many loci

that influence resource acquisition (Rowe and Houle 1996). The expression of condition-dependent sexual traits should therefore "capture" and magnify the negative effects of inbreeding status on life-history traits that affect resource acquisition (Prokop et al. 2010). There is, indeed, evidence that sexually selected male traits favored during mate choice (e.g., de Boer et al. 2016) or fights (e.g., Sartori and Mantovani 2013) are negatively affected by the bearer's inbreeding status.

To date, there have been few attempts to compare the extent of inbreeding depression on different sexually selected traits. Sexual selection usually involves both pre- and postcopulatory phases (e.g., Devigili et al. 2015). Male reproductive success partly depends on the ability to inseminate females, generating precopulatory sexual selection for ornaments, displays, and weapons that function to attract or defend mates. But reproductive success also depends on the ability to convert insemination into fertilization, driving postcopulatory sexual selection for ejaculate traits and sperm performance (Parker and Pizzari 2010). The predicted effect of male inbreeding status on traits under precopulatory sexual selection is straightforward—it is expected to lead to reduced trait expression. The relationship between heterozygosity and sperm traits is more complex, however, because sperm are haploid. Inbreeding depression for sperm traits, such as motility, presumably arises through effects of male inbreeding status on associated diploid cells (Nayernia et al. 1996), for instance, germ cells that create sperm, and secretory cells that produce seminal substances that affect sperm performance. Losdat et al. (2014) recently reviewed the evidence that inbreeding negatively affects quantitative ejaculate measures (i.e., sperm count, ejaculate volume): inbreeding depression occurs in some species (e.g., Zajitschek and Brooks 2010; Maximini et al. 2011; Fox et al. 2012) but not others (e.g., Aurich et al. 2003; van Eldik et al. 2006). Losdat et al. also highlighted evidence for inbreeding depression in sperm morphology and motility in some species (e.g., Asa et al. 2007; Malo et al. 2010; Opatová et al. 2016), but, again, not in others (e.g., Okada et al. 2011; Ruiz-López et al. 2012; Gasparini et al. 2013).

Do these reported effects of inbreeding on sperm and ejaculate traits actually translate into lower fitness? And is there postcopulatory sexual selection against inbred males due to sperm competition (Losdat et al. 2014)? Interestingly, several studies that found no detectable differences in sperm quality or quantity between inbred and outbred males still reported that inbred males gain less paternity under sperm competition (e.g., *Tribolium castaneum*, Michalczyk et al. 2010; *Mesocricetus auratus*, Fritzsche et al. 2006). This suggests that despite no detectable effects of inbreeding status on ejaculate traits (either because measurable differences are small relative to measurement error, or because researchers are measuring the wrong traits), inbreeding depression can still occur due to sexual selection because of sperm competition. Despite this, few studies have used controlled experiments to investigate how inbreeding affects male fertilization success (Simmons 2011; Losdat et al. 2014).

To understand how inbreeding affects sexual selection requires studies that quantify its effects on different male traits and fitness components. Field studies of inbreeding depression are valuable but, strictly speaking, we have to manipulate heterozygosity levels experimentally, usually via controlled breeding designs, to identify causality (e.g., Slate and Pemberton 2006; see commentary in Opatová et al. 2016). Here, we do this using the mosquitofish, *Gambusia holbrooki*. In an earlier study, we showed that natural variation in heterozygosity predicts a male's share of paternity when wild-caught males compete for females in glasshouse ponds (Head et al., in press). In a second study, we experimentally created inbred (full-sib matings) and outbred males and again showed that inbred males had significantly lower reproductive success than outbred males when they freely competed for females (Vega-Trejo et al. 2017). In both experiments natural selection was effectively eliminated because males entered the mating pool as adults. This rules out potential effects of a male's inbreeding status on mortality affecting his success, as $<2\%$ of males died during the mating period. The lower success of inbred males is therefore, by definition, attributable to sexual selection: nonrandom variation in male reproductive success is due to competition for mates to gain insemination opportunities and sperm competition to fertilize eggs. This raises an obvious question: which component of sexual selection has a greater effect on male success: mating rate or sperm competitiveness?

We investigated several possible causes of the lower reproductive success of inbred male *G. holbrooki*. First, we quantified the effect of inbreeding status on precopulatory sexual selection, by examining male attractiveness to females. Second, we tested for postcopulatory sexual selection against inbred males due to inbreeding depression for ejaculate traits (sperm number) and sperm traits (swimming velocity). Finally, we use artificial insemination to test directly whether a male's inbreeding status lowers his fertilization success in a controlled setting. Our initial breeding design to generate inbred and outbred fish was set up to test whether inbreeding affects an individual's ability to compensate for a poor rearing environment (Vega-Trejo et al. 2016a). To this end, the sperm data presented here has been analyzed previously (Vega-Trejo et al. 2016b), albeit with a different question in mind (i.e., the effect of rearing environment, but not inbreeding). The data we present on male attractiveness and paternity was collected solely for the current study. Here, for simplicity, we only analyze the effects of inbreeding. In the Supporting Information, we show that rearing environment did not affect the focal traits, or share of paternity.

Methods

STUDY SPECIES

Gambusia holbrooki exhibits sexually coercive mating behavior whereby males consistently pursue and attempt to copulate with females. However, mate choice also occurs because females prefer to associate with large males that have a relatively long gonopodium (intromittent organ modified from the anal fin) (Kahn et al. 2009). Both of these male traits have previously been shown to predict insemination success (Head et al. 2015b). As for traits involved in postcopulatory sexual selection, evidence from another poeciliid, the guppy (*Poecilia reticulata*), suggests that sperm number and velocity are both predictors of fertilization success under sperm competition (Boschetto et al. 2011).

ORIGIN AND MAINTENANCE OF EXPERIMENTAL FISH

Our laboratory stock was collected in Canberra, Australia (mosquitofish were introduced to Australia in the 1920's; Ayres et al. 2010) from ponds that are less than 20 years old and likely to have been naturally colonized from a nearby artificial lake that was built in 1974. The mean heterozygosity for these wild fish is 0.27 (based on 3171 SNP loci: Head et al., in press). This is within the range of mean heterozygosity (based on SNP data) (0.23–0.63) reported for endemic mosquitofish in the southern United States (Vera et al. 2016).

The breeding design we used to experimentally create inbred and outbred fish has been fully detailed elsewhere (Vega-Trejo et al. 2015, 2016a) so we provide only a brief description here. We collected wild, gravid females and raised their offspring in single-sex tanks to ensure their virginity. All fish were kept on a 14-h light:10-hdark cycle at 28°C, and fed ad libitum with *Artemia* nauplii and commercial flakes. Once they had matured, males and females were randomly paired to create 58 full sibling families and the offspring from these families were then used in a fully balanced breeding design to create inbred and outbred experimental fish. We set up 29 blocks that each had mating individuals from two families (i.e., A and B). Brothers and sisters were paired to create inbred offspring (AA and BB), whereas males and females from opposite families were used in reciprocal pairings to create outbred offspring (AB and BA). Fish from these broods were individually reared in 1 L tanks on one of two diets, but including rearing diet in our analyses did not influence our main findings with respect to inbreeding depression (details in the Supporting Information). All data have been made available on Dryad (Marsh et al. 2017).

Experiment 1: Do females prefer to associate with outbred males?

We used two-choice tests to determine whether females spent more time with outbred than inbred males. We matched pairs of inbred and outbred males for size (tolerance < 1 mm) (inbreeding does not affect adult male size; Vega-Trejo et al. 2016a) and diet type. Trials took place in a 16 L tank ($38 \times 19 \times 19$ cm^3) divided into three sections: two end sections ($5 \times 19 \times 19$ cm^3), each housing a male, and a central section ($28 \times 19 \times 19$ cm^3) for the female (see Vega-Trejo et al. 2014). The sections were separated by a removable opaque screen (to minimize visual and olfactory contact before the trial) and by a mesh screen (made from tulle netting). To begin a trial, one male was randomly assigned to each end of the tank (but across all trials inbred and outbred males were at each end equally often) and a virgin stock female was placed in the central compartment. Fish were allowed to acclimate for 15min, after which we removed the opaque screens. Behavioral observations lasted 10 min during which we recorded female association time with each male (i.e., < 4 cm from a male's compartment). If a female did not visit the "association zone" of each male at least once ($N = 8$ of 117 trials), the same pair of males were then retested with a second female. We ran 109 successful trials. Our measure of association time predicts copulation attempts by male *G. holbrooki* in free swimming trials (Vega-Trejo et al. 2014). Data were collected blind to male inbreeding status.

Analysis

To test whether females preferred to associate with outbred males, we ran a generalized linear model (GLM) with quasibinomial error to account for overdispersion. We linked the amount of time the female spent with the outbred male and the amount of time spent with the inbred male and used this as the response variable in our binomial model. This can be broadly interpreted as the proportion of time spent with the outbred male, weighted by the total time spent with both males. Our key test is whether on the underlying latent scale the intercept differs from 0. This is equivalent to asking if the proportion of time a female spent with the outbred male is significantly greater than 50%, indicating a preference for outbred males.

Experiment 2: Do outbred males produce more sperm, or better performing sperm?

We collected sperm on three occasions. On the first occasion (Day 1), we stripped virgin males of sperm to measure their maximum sperm reserves. One day later (Day 2), we stripped males to estimate their sperm replenishment rate (i.e., sperm production in 24 h). Males do not fully replenish their sperm reserves until at least three days after being stripped (O'Dea et al. 2014), so using the number of sperm stripped on Day 2 is a valid measure of the replenishment rate. On the next day (Day 3), we stripped males to measure sperm velocity.

We stripped sperm following the methods of Matthews et al. (1997). Full details of our methods are in Vega-Trejo et al. (2016b), so we describe them only briefly here. Following

anesthetization in iced water, we placed males on their side under a dissecting microscope. We swung the gonopodium forward and at its base we applied gentle pressure to the abdomen so that the ejaculate was released into 100 μL of saline solution. We transferred the ejaculate to an Eppendorf tube with 100–900 μL of extender medium depending on the amount of ejaculate stripped. Males were then returned to their tanks.

To estimate the number of sperm, we thoroughly mixed the sample and then placed 3 μL of the solution on a 20 μm capillary slide (Leja) and counted the sperm using a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) under 100 × magnification. We counted five subsamples per male.

To estimate sperm velocity, we analyzed three samples per ejaculate per male. For each sample, we collected 3 μL of diluted sperm (see above) and placed it in the center of a cell of a 12-cell multitest slide (MP Biomedicals, Aurora, OH, USA). The sample was then activated and covered with a cover slip. We analyzed sperm velocity within 30 sec of activation. We measured 109.3 ± 3.49 SE sperm tracks per ejaculate. We recorded two standard measures of sperm velocity: (1) average path velocity (VAP), which estimates the average velocity of sperm cells over a smoothed cell path and (2) curvilinear velocity (VCL), the actual velocity along the trajectory. Due to a near perfect correlation of VAP with VCL ($r = 0.961$, $P < 0.001$), we only use VAP in our analyses.

We also measured male body size one week after the sperm extractions. Males were anesthetized by submersion in ice water, placed on their side, and photographed. We measured standard length (SL = snout tip to caudal fin base) in *Image J* (Abramoff et al. 2004). Data were collected blind to male inbreeding status.

Analysis

We measured maximum sperm reserves, sperm replenishment rate, and sperm velocity for 452 males (inbred = 224, outbred = 228). These data have been analyzed elsewhere with a focus on the hidden costs of compensatory growth (i.e., only analyzing the effect of diet; Vega-Trejo et al. 2016b). Here, we are interested in how inbreeding affects sperm traits.

To analyze the effect of inbreeding, we used generalized linear mixed models (GLMMs). We ran separate models for sperm at Day 1, Day 2 (i.e., replenishment rate) and sperm velocity (VAP). In each model, inbreeding status was a fixed effect and male standard length was a covariate to control for size-dependent variation in testes size, hence sperm reserves. Adult male size does not depend on his inbreeding status (Vega-Trejo et al. 2016a,b). In all GLMMs, we specified a Gaussian error structure and checked the distribution of model residuals to ensure this was appropriate. Gaussian error structure was chosen over Poisson errors (for count data) because the latter were highly overdispersed. Each model was fitted in *R* using the *lme4* package, with block, mother, father, and male ID as random factors. Model terms were tested for significance using the *Anova* function in the *car* package specifying Type III Wald chi-square tests so that the effect of inbreeding is estimated after controlling for body size.

We also calculated the inbreeding load (calculated as [Ln(Inbred mean/Outbred mean)]/0.25) following Losdat et al. (2014) to allow comparison of the effects of inbreeding status on male sperm traits with a previously published meta-analysis (Losdat et al. 2014). The inbreeding load was calculated for each block and then averaged across the 29 blocks. These are presented in Table 1.

Experiment 3: Do outbred males sire more offspring with sperm competition?

To determine whether outbred males have more competitive sperm, we artificially inseminated females with equal numbers of sperm from an outbred and an inbred male (matched as in Experiment 1). We then calculated their share of paternity.

To inseminate females, we first anesthetized each male in ice water, and stripped sperm as outlined in Experiment 2. We transferred 20 sperm bundles (in 3μL saline solution) from each male into microcentrifuge tubes that contained an additional 3 μL of saline solution, so that the tube contained 40 sperm bundles (20 per male). We then repeated this procedure to have two replicates per male pair. We allowed the sperm bundles to settle (ensuring we were able to collect all bundles) and used a micropipette

Table 1. The effect of inbreeding status on male sperm traits.

	Outbred			Inbred					
Trait	*N*	Mean	SE	*N*	Mean	SE	Mean Inbreeding Load	χ^2	P
Sperm number ($\times 10^{-5}$)	225	187.46	5.74	228	182.90	6.74	–0.024	0.094	0.759
Sperm replenishment ($\times 10^{-5}$)	225	56.41	3.01	228	55.47	2.82	–0.092	0.293	0.589
Sperm velocity	196	82.40	1.14	198	83.35	1.21	0.010	0.143	0.706

The inbreeding load following Losdat et al. (2014) (see Methods).

to draw 3 μL of the solution from the bottom of the tube. This solution, containing the intact sperm bundles from both males, was inserted into the reproductive tract of an anesthetized female. This process was carried out for both tubes so that each pair of males artificially inseminated two females. Males were preserved in ethanol for genotyping.

Following insemination, females were transferred into individual 1 L tanks containing a mesh divider and plastic plants to provide a refuge for offspring. Females were fed thrice daily with *Artemia* nauplii and checked twice daily for offspring. Offspring were collected, euthanized, and preserved in ethanol for genotyping. We continued checking for offspring for six weeks following insemination. In this time females could produce up to two broods.

To determine paternity, we took tissue samples from each male (124 males from 62 pairs) and all available fry ($n = 492$ offspring). For adults, DNA was extracted from the tail muscle/caudal fin. For offspring, DNA was extracted from the whole body (excluding head). DNA extraction and genotyping were performed by a commercial service (DiversityArray) using DArTseq (Elshire et al. 2011; Kilian et al. 2012; Courtois et al. 2013; Cruz et al. 2013; Raman et al. 2014). Full genotyping methodology details are in Booksmythe et al. (2016).

Genotyping was unsuccessful for only two of the 492 offspring, who were excluded from further analysis. After cleanup, our data yielded 2138 SNPs (average call rate 98.8%, average reproducibility rate 96.4%), which were used to calculate a Hamming Distance Matrix for the remaining 614 fish. Recent studies show that as few as 30 optimized SNPs are sufficient to differentiate among 100,000 individuals based on Hamming Distance values (Hu et al. 2015). In our study, we used a large number of high-quality SNPs to assign paternity between only two possible sires. The Hamming Distances between an offspring and its two possible fathers was compared, and the male with the highest genetic similarity (lowest distance) to an individual fry was assigned the paternity. We confidently assigned paternity to 488 fry but two fry appeared to be unrelated to either male: both had a Hamming distance similar to that between the focal fry and males from other test pairs (i.e., males that could not have been sires). These two fry were attributed to contamination during the insemination process, and excluded from analysis. We have elsewhere shown that assigning parentage at birth does not result in a downward bias in our estimate of inbred males' share of paternity due to greater embryo mortality of offspring sired by an inbred male (Vega-Trejo et al. 2017).

Using the SNP data, we also calculated the heterozygosity of each fish as the number of SNP markers that were scored as heterozygous divided by the total number of successfully classified markers for that fish (F_{het}). This is essentially a measure of genome wide heterozygosity, and F_{het} is identical to $1 - F_{hom}$, as used by Bérénos et al. (2016). Data were collected blind to male treatment.

Analysis

We analyzed the paternity data in two ways. First, we tested for an effect of inbreeding on the proportion of offspring sired by the outbred male using inbreeding status defined by our breeding design (i.e., a fixed categorical factor). We conducted this analysis because it was how we originally planned it, and to be comparable with analyses in our other experiments. We used a GLM with a quasibinomial error structure (to account for overdispersion). The proportion of offspring sired by the outbred male in each pair was created using the *cbind* function in *R* to weight each pair by the total number of offspring sired. Here, we pooled offspring from different broods (whether produced consecutively by the same mother and/or by both females) to avoid psuedoreplication. Because there were few pairs for which two females gave birth (8 out of 62) or for which females had multiple broods (2 out of 62), there was insufficient within-pair replication to justify a more complex model treating male pair as a random factor.

Second, we tested how relative heterozygosity influenced paternity. We did not originally intend to conduct this analysis because we were unaware this information would be available. We formally state this to distinguish between planned and exploratory data analysis (see Head et al. 2015a). In hindsight, however, this might be a better test for inbreeding depression because inbreeding is expected to reduce fitness *due to* lower heterozygosity (but see *Discussion*). Our breeding design created inbred and outbred males with significantly different mean heterozygosity (based on >2000 loci; see *Results*). Even so, the difference in heterozygosity between paired males varied (outbred–inbred difference: range –0.06 to 0.19; in 5 of 62 cases the outbred male was less heterozygous). We again used a GLM but with the weighted proportion of offspring sired by the more heterozygous male as our response variable and relative heterozygosity (high heterozygosity – low heterozygosity)/high heterozygosity) as the predictor. Using relative heterozygosity rather than the absolute difference in heterozygosity assumes that the effect of the difference in heterozygosity is nonlinear with respect to absolute heterozygosity. That is, the effect of a 0.1 difference in heterozygosity is expected to be greater for pairs with low absolute heterozygosity than it is for those with high absolute heterozygosity. Analyzing the data using the absolute difference in heterozygosity as the covariate gives qualitatively similar results (see *Results*).

Results

Based on data from over 2000 SNP loci, we found that inbred males had significantly lower mean heterozygosity than outbred males (mean ± SE – F_{het} inbred: 0.262 ± 0.005; F_{het} outbred:

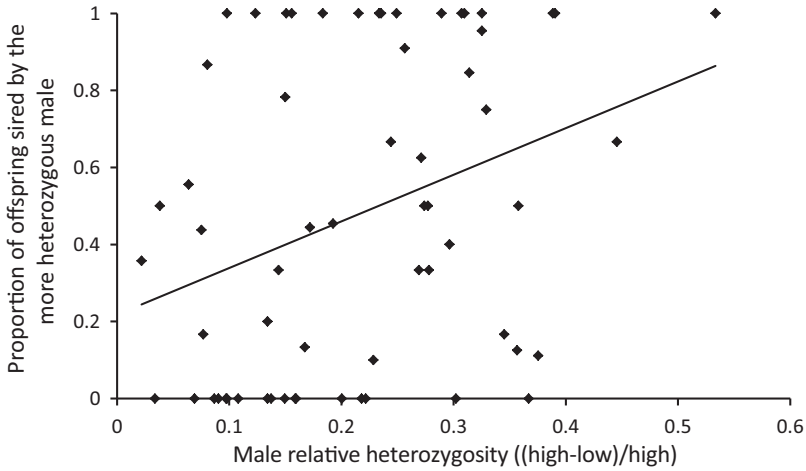

Figure 1. The relationship between male relative heterozygosity and the proportion of offspring sired by the more heterozygous male.

0.332 ± 0.005, $F_{(1,122)} = 99.99$, $P < 0.001$). The mean within-pair difference in heterozygosity of 20.1% is less than the 25% reduction expected from full-sibling matings in a fully outbred population (95% confidence intervals: 16.6–23.6%). This discrepancy might be attributable to early embryo mortality of more homozygous males (see Vega Trejo et al. 2015). It should also be noted that in another sample of inbred and outbred males from the same breeding design, the decline in heterozygosity was 23.2% (see Vega Trejo et al. 2017), which is closer to the expected 25% decline (see *Discussion*).

EXPERIMENT 1: DO FEMALES PREFER TO ASSOCIATE WITH OUTBRED MALES?

Females spent 45.5% (±2.0 SE) of the trial associating with a male, and they spent 48.7% (±3.2 SE) of their association time with the outbred male. This was not significantly different from 50% (intercept: $t = 0.41$, $P = 0.683$).

EXPERIMENT 2: DO OUTBRED MALES PRODUCE MORE SPERM, OR BETTER PERFORMING SPERM?

Outbred and inbred males did not differ significantly in their maximal number of sperm, sperm replenishment rate, or sperm velocity (Table 1). Larger males had significantly more sperm ($\chi^2 = 4.371$, $P = 0.037$) and significantly faster sperm ($\chi^2 = 4.070$, $p = 0.044$), but there was no relationship between male size and replenishment rate ($\chi^2 = 0.158$, $p = 0.691$).

EXPERIMENT 3: DO OUTBRED MALES SIRE MORE OFFSPRING WITH SPERM COMPETITION?

Outbred males sired 55.5% (±4.85) of offspring, which was not significantly different from 50% (intercept: $t = 1.128$, $P = 0.264$). However, a greater relative heterozygosity difference between paired males was associated with a significant increase in the share of paternity by the more heterozygous male ($t = 2.350$, $P = 0.022$) (Fig. 1). The same was true if we used the absolute difference in heterozygosity between the two males ($t = 2.437$, $P = 0.018$).

Discussion

It is widely accepted that mating with relatives can reduce the fitness of parents because they produce less fit offspring who suffer from inbreeding depression due to their increased homozygosity. However, the relative magnitude of the effect of being inbred on specific types of traits is often unclear. This is partly because nonexperimental studies of wild populations can rarely disentangle the potential causes of reduced fitness. For *G. Holbrooki*, we have previously shown that low heterozygosity (Head et al., in press) and inbreeding status (generated from controlled breeding) (Vega-Trejo et al. 2017) are associated with lower male reproductive success due to sexual selection. But it is not known why these males are less successful. In the present study, we quantified how inbreeding status affects male attractiveness, ejaculate traits, and share of paternity under sperm competition. To infer causality, we took an experimental approach to both the generation of variation in levels of inbreeding (i.e., used a breeding design) and how we measured male attractiveness and fertilization ability (i.e., two choice trials and competitive artificial inseminations).

ATTRACTIVENESS

Females in two-choice mating trials did not associate preferentially with outbred males. This contrasts with similar experiments in other species that have shown that inbred males are less attractive (e.g., in mice—Thom et al. 2008; Ilmonen et al. 2009; mealworm beetles—Pölkki et al. 2012; field crickets—Drayton et al. 2010; fruit flies—Okada et al. 2011; and butterflies—van Bergen et al. 2013). Other studies have, however, failed to

report inbreeding depression for male attractiveness (e.g., Michalczyk et al. 2010). Why do only some studies find female preferences for outbred males? The obvious explanation is that female mate choice decisions in some species are based on traits unaffected by reduced heterozygosity. Indeed, we have shown previously in *G. holbrooki* that inbreeding affected neither male body size, nor gonopodium length (Vega-Trejo et al. 2016a, b), two traits that influence female mate choice (Kahn et al. 2009) and male insemination success (Head et al. 2015b). However, even in the absence of direct effects of inbreeding status on sexual traits, there may still be benefits to females that avoid inbred males. Although it is often assumed that heterozygosity is not heritable (i.e., that mating with an inbred male will not lead to inbreeding depression in the resultant offspring), this is not always the case (Mitton et al. 1993; Nietlisbach et al. 2016). Heritability of heterozygosity occurs whenever allele frequencies are asymmetric (e.g., when allele frequencies are not 50:50 for biallelic loci; Mitton et al. 1993). That said, another explanation for the lack of a female preference for outbred males is simply that the study population (or its source population) has been sufficiently large over recent evolutionary time that the risk of inbreeding is low under random mating in most situations, so there is no meaningful selection on females for inbreeding avoidance.

SPERM TRAITS

We found no inbreeding depression for maximum sperm reserves, sperm replenishment rate, or sperm velocity. Losdat et al. (2014) reported that of 99 sperm traits examined in 24 species, a significant negative effect of inbreeding occurred in 48 cases, no detectable effect in 50 cases, and a significantly positive effect in one case. The general trend is therefore that inbreeding negatively affects sperm production and motility. The mean inbreeding load reported by Losdat et al. (2014) for sperm traits was –0.129 (95% CI: –0.209 to –0.049). How does this compare with our findings? In Table 1, we provide inbreeding loads for the sperm traits measured here—two of three are outside the 95% confidence intervals calculated from Losdat et al. (2014). Given our large sample sizes, it therefore seems likely that the lack of inbreeding that we report for these traits is a true null effect, and that our findings are anomalous with respect to the general pattern seen across many species.

Of course, sperm and ejaculate traits other than those we measured might be negatively affected by inbreeding. For example, many studies have shown that inbreeding creates sperm abnormalities (e.g., studies in Fitzpatrick and Evans 2009; see also Opatová et al. 2016). However, regardless of whether changes in sperm morphology or sperm count associated with inbreeding are detected, the evolutionary question is whether inbred males have lower fertilization success under sperm competition. It is necessary to formally test for this to determine whether postcopulatory sexual selection acts against inbred males.

SPERM COMPETITIVE ABILITY: PATERNITY

Our use of artificial insemination controls for any variation in the ability of males to inseminate females and potential differences in the number of sperm males transfer. The interpretation of our results depends on the exact statistical analysis. Based on our pedigree, outbred males sired more offspring than inbred males (mean share of paternity of 55.5%), but this was not statistically significant, implying that inbred males do not have less competitive ejaculates. However, because the use of SNPs provided data on the proportion of heterozygous loci (>2000 SNPs) for each male, we ran a post hoc exploratory test using this as another proxy for inbreeding (see discussion in Szulkin et al. 2010; Bérénos et al. 2014). Heterozygosity and inbreeding status are correlated in our sample ($r = 0.66$, $P < 0.001$). Even so, the share of paternity gained by the more heterozygous male increased with the magnitude of the relative (or absolute) difference in heterozygosity between the two competing males. This implies that heterozygosity, hence being more inbred, is under postcopulatory sexual selection because it reduces a male's competitive fertilization ability. Which of these two proxies provide a better estimate of an individual's overall level of heterozygosity is debatable. Mendelian sampling variance that affects the heterozygosity levels of individuals means that realized heterozygosity always varies around that expected based on the breeding design (i.e., a pedigree based inbreeding coefficient). This fact seemingly makes a SNP-based estimate more accurate, because it provides information about observed rather than average expected heterozygosity (see Visscher et al. 2006 for empirical data showing the effect of Mendelian sampling). The counterargument, albeit a Weak one, is that our SNP-based estimate will show sampling error, and might show a sampling bias, because it is based on a subset of markers, while the inbreeding coefficient is an unbiased genome-wide expectation of heterozygosity. One puzzle is why there was not a stronger correlation between the inbreeding coefficient (based on our experiment) and the observed level of SNP heterozygosity. There is clearly higher variation in homozygosity in the outbred population than expected given random mating in a single, large population. This is presumably due to recent variation in the extent of shared ancestry among wild individuals, which could arise due to, for example, periodic arrival of fish from small, isolated populations where inbreeding occurs more often. In general, future research testing how well different proxies of heterozygosity (e.g., pedigree-based inbreeding coefficients versus direct measures of heterozygosity) predict fitness would be useful (see Nietlisbach et al. 2017 for one such comparison).

Conclusions

Two previous studies showed that inbred (or less heterozygous) male *G. holbrooki* gain a smaller share of paternity when they compete for females, which we attribute to sexual selection against inbred males (Head et al., in press; Vega-Trejo et al. 2017). This could be due to a reduced ability to inseminate females and/or to lower ejaculate competitiveness. Here, we found no evidence that females are less likely to associate with inbred males. This suggests that inbred males are unlikely to have a lower mating rate due to reduced attractiveness, as occurs in some species (e.g., van Bergen et al. 2013). This does not, however, exclude other forms of precopulatory sexual selection. A male's mating rate in *G. holbrooki* also depends on his ability to: chase away rivals (e.g., Bisazza and Marin 1991), sneak up on females (e.g., Pilastro et al. 1997), and the success of the resultant insemination attempts (e.g., Head et al. 2015b). There was also no evidence that inbreeding affected sperm number or motility, despite our large sample size ($N > 400$ males). There was, however, evidence that less heterozygous (i.e., more inbred) males have less competitive sperm (Fig. 1). This implies that lower competitive fertilization ability might partly explain the lower paternity of inbred males in our earlier studies. If true, this raises the challenge of identifying which aspects of the ejaculate or of the sperm themselves are affected by inbreeding. Potential candidates include sperm longevity and the speed with which sperm penetrate an egg. Although our results suggest that postcopulatory sexual selection could be more important than mate choice in selecting against inbred male *G. holbrooki*, further studies on other components of precopulatory sexual selection, particularly in a competitive setting, are needed before general conclusions can be made about the relative role of pre- and postcopulatory sexual selection selecting against inbreeding.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Effects of inbreeding and diet on male sperm traits.