RESEARCH ARTICLE

Sexual selection on male body size, genital length and heterozygosity: Consistency across habitats and social settings

Megan L. Head [iD] | Andrew T. Kahn | Jonathan M. Henshaw | J. Scott Keogh | Michael D. Jennions

Division of Ecology and Evolution, Research School of Biology, The Australian National University, Canberra, Australia

Correspondence
Megan L. Head
Email: megan.head@anu.edu.au

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Abstract

1. Spatial and temporal variation in environmental factors and the social setting can help to maintain genetic variation in sexually selected traits if it affects the strength of directional selection. A key social parameter which affects the intensity of, and sometimes predicts the response to, mating competition is the operational sex ratio (OSR; ratio of receptive males to females).

2. How the OSR affects selection for specific male traits is poorly understood. It is also unclear how sexual selection is affected by interactions between the OSR and environmental factors, such as habitat complexity, that alter key male–female interactions such as mate encounter rates.

3. Here, we experimentally manipulated the OSR and habitat complexity and quantified sexual selection on male mosquitofish (*Gambusia holbrooki*) by directly measuring male reproductive success (i.e. paternity).

4. We show that despite a more equitable sharing of paternity (i.e. higher levels of multiple paternity) under a male-biased OSR, selection on focal male traits was unaffected by the OSR or habitat complexity. Instead, sexual selection consistently, and significantly, favoured smaller bodied males, males with higher genome wide heterozygosity (based on >3,000 SNP markers) and males with a relatively long gonopodium (intromittent organ).

5. Our results show that sexual selection on male body size, relative genital size and heterozygosity in this system is consistent across environments that vary in ecological parameters that are expected to influence mate encounter rates.

KEYWORDS
body size, environmental heterogeneity, HFC, poeciliid, reproductive success

1 | INTRODUCTION

Variation in the strength and form of sexual selection has generated enormous diversity in morphology, behaviour and physiology between the sexes, across populations and among species (Pfennig & Pfennig, 2010). Field studies have shown that sexual selection can vary across populations and over time (e.g. Kasumovic, Bruce, Andrade, & Herberstein, 2008; Wacker, Amundsen, Forsgren, & Mobley, 2014).

Spatio-temporal variation is thought to be important to maintain genetic diversity (e.g. Ellner & Hairston, 1994; Felsenstein, 1976) and, more specifically, it is thought to slow the erosion of additive genetic variation for sexual traits (Day, 2000; Holman & Kokko, 2014) that are usually under strong unidirectional selection (Hoekstra et al., 2001). Variation in the direction of sexual selection across heterogeneous environments, and genotype-by-environment effects, is often posited as a partial explanation for the persistence of additive genetic variation

in sexual traits (Hunt & Hosken, 2014), but relatively few studies address this question by taking an experimental approach to manipulating the environment (Cornwallis & Uller, 2010).

Sexual selection on focal traits is known to vary among populations or between breeding cycles, but we rarely understand why (Janicke, David, & Chapuis, 2015). Most research on variation in sexual selection involves long-term observational studies of wild populations that draw inferences based on annual or seasonal changes in ecological or social factors that can plausibly be linked to shifts in mate availability (e.g. Kasumovic et al., 2008; Wacker et al., 2014). Unfortunately, many ecological and social parameters covary in nature so it is difficult to identify which parameters actually drive variation in sexual selection. Similar problems arise when comparing populations to try to identify factors that affect sexual selection. For example, population differences in male guppy (*Poecilia reticulata*) coloration are attributed to habitat differences in predation (e.g. Endler, 1980), but predation also affects demography (e.g. Arendt, Reznick, & Lopez-Sepulcre, 2014), which could independently affect selection on male sexual signals. Ultimately, experiments are required to determine whether specific environmental or social factors moderate selection for specific traits.

Sexual selection studies often focus on the role of the social environment, especially the operational sex ratio (OSR: the ratio of sexually receptive males to females) (review: Kvarnemo & Ahnesjo, 1996; Shuster, 2016). The OSR is, by definition, a measure of the intensity of competition for mates, but its value in predicting the strength of sexual selection is much debated (e.g. Clutton-Brock & Parker, 1992; Klug, Heuschele, Jennions, & Kokko, 2010). Variance in male reproductive success (i.e. the opportunity for selection) is expected to increase when the OSR is more male-biased due to greater competition for mates (Emlen & Oring, 1977; Kvarnemo & Ahnesjo, 1996; Shuster, 2016). This is not inevitable, however, as it depends on idiosyncratic features of each species' biology (e.g. is harem defence easier or harder as the OSR becomes more biased?) (Jennions, Kokko, & Klug, 2012; Klug et al., 2010; meta-analysis: Moura & Cardoso Peixoto, 2013). The OSR might affect the various components of sexual selection in different ways so that the observed effects depend on the type of mating system, and which traits were measured (Kokko & Rankin, 2006). For example, Weir, Grant, and Hutchings (2011) noted that as the OSR becomes more biased the competitive sex tends to become more aggressive, but courts less. This will lead to different estimates of how the OSR affects selection on sexual traits depending on whether one measures traits involved in intra- vs. intersexual selection (Fitze & Le Galliard, 2008; Head, Lindholm, & Brooks, 2008 and references therein). Finally, inconsistent effects of the OSR on sexual selection might arise because its effects are context-dependent and modulated by other environmental factors (e.g. predation risk).

One ecological parameter that is of special interest is habitat complexity, because it can have profound effects on sexual selection (Myhre, Forsgren, & Amundsen, 2013). For example, the transmission of mating signals depends on the habitat type: open habitats generally allow transmission of signals over greater distances. Such differences affect how females perceive and assess male sexual signals and alter selection on males (e.g. sensory bias/drive—Boughman, 2002; Endler

& Basolo, 1998). Habitat complexity can also affect selection on traits that affect male fighting success. For example, recent studies report higher aggression between male sticklebacks (*Gasterosteus aculeatus*) in more open habitats that might generate stronger selection for larger body size than in closed habitats (Lackey & Boughman, 2013). Finally, the OSR and habitat complexity are both likely to alter mate encounter rates, and habitat complexity can generate small-scale variation in local OSRs and densities (i.e. those directly experienced by a female) (Myhre et al., 2013). Such effects could alter the intensity of male–male competition and opportunities for female choice. For example, in guppies greater habitat complexity reduced interference competition between males and increased female mating receptivity (Hibler & Houde, 2006).

Here, we conduct an experiment to investigate how habitat complexity and the adult sex ratio (which for convenience we refer to as the OSR because male mosquitofish constantly mate with females) affect sexual selection on male mosquitofish *Gambusia holbrooki*. This species is well suited to test how these two factors affect sexual selection because it inhabits habitats that vary greatly in their structural complexity (Pyke, 2005), and adult sex ratios show major shifts over the breeding season (Kahn, Kokko, & Jennions, 2013). We used SNP-based paternity analysis to determine male reproductive success and thereby quantify sexual selection on three focal male traits that have previously been identified as putative targets of sexual selection (male body size—McPeek, 1992; Pilastro, Giacomello, & Bisazza, 1997: residual gonopodium length—Head, Vega-Trejo, Jacomb, & Jennions, 2015; Kahn, Mautz, & Jennions, 2010: and genomewide heterozygosity—Vega Trejo, Head, Keogh, & Jennions, 2017).

In mosquitofish, males incessantly attempt to mate by approaching females from behind and thrusting their gonopodium (a modified anal fin used to transfer sperm) into her gonopore (Bisazza, 1993; Bisazza & Marin, 1995). Male size and residual gonopodium size are expected to be important determinants of reproductive success because large males can dominate access to females (Bisazza & Marin, 1991), and female mate choice (in the form of longer association times) seems to favour larger males with a relatively long gonopodium (Bisazza, Vaccari, & Pilastro, 2001; Head et al., 2015; Kahn et al., 2010; McPeek, 1992). However, smaller males are more adept at sneaking copulations with females (Pilastro et al., 1997). These contrasting selection pressures suggest that sexual selection on male traits, resulting from the balance of female mate choice and male sexual coercion, might depend on the social setting. For instance, as populations become more male-biased selection might tilt in favour of male ability to sneak copulate and favour smaller over larger males and weaken selection for a longer gonopodium, as female choice becomes less important. In contrast, complex habitats might make it easier for females to evade sexual coercion and to exert mate choice such that overall sexual selection might be expected to favour large males with a long gonopodium. Finally, experimental inbreeding studies show that heterozygosity is positively correlated with male reproductive success (Vega-Trejo et al. 2017), but the effect of natural, standing variation in heterozygosity is unknown, as are the specific traits whose expression is negatively affected by a decline in heterozygosity (i.e. the proximate mechanisms

driving sexual selection against inbred males). Here we aim to directly quantify how OSR and habitat complexity affect sexual selection on these traits, and to determine whether differences in habitat complexity mediate the potential effects of the OSR on sexual selection.

2 | MATERIALS AND METHODS

2.1 | Experimental design

We independently manipulated the adult sex ratio and habitat complexity in pools (1 m diameter, 15 cm depth) in a greenhouse using a 2 × 2 factorial design. We had two levels of habitat complexity. In the "simple" habitat the pool floor was lined with gravel, the pool walls were lined with white plastic, and there was no vegetation or cover. The "complex" habitat was the same, but we added a network of white plastic baffles to create multiple, interconnected compartments (Figure S1). Manipulating habitat complexity in this way ensured that the manipulation was applied evenly across the pool. The manipulation is similar to that used by Hibler and Houde (2006) who found that increased visual isolation in complex habitats altered sexual behaviour in guppies. We also had two levels of OSR. The female-biased OSR consisted of 10 males and 20 females in a pool. The male-biased OSR consisted of 10 males and 5 females in half a pool. We avoid confounding changes in fish density and the number of males by adjusting the pool size to keep the number of males and the overall density of fish constant. Fish density across all treatments was *c*. 1 fish per 4 L, which falls well within *G. holbrooki* densities in the wild (e.g. Jordan, Babbitt, & McIvor, 1998) and those used in previous studies of other poeciliid fishes (e.g. Devigili, Evans, Di Nisio, & Pilastro, 2015; Hibler & Houde, 2006; Mariette, Zajitschek, Garcia, & Brooks, 2010). Any treatment differences are therefore due to the OSR and/or habitat complexity, and not to demographic parameters that often covary with OSR (see Head et al., 2008). We set up six blocks: each comprised one replicate per treatment ($n = 6$ blocks × 4 treatments × 10 males = 240 males in 24 replicates). Our experimental design was sufficient to detect medium to large effect sizes (see Section 3).

2.2 | Experimental protocol

We used *G. holbrooki* from ponds in Canberra, Australia (35°14′27″S, 149°5′27″E and 35°14′13″S, 149°5′55″E). These ponds are less than 2 km apart and likely to be connected during periods of high rainfall. Experimental males were caught from the wild. Experimental females were laboratory-reared offspring of wild-caught females. This ensured they were virgins at the beginning of our experiment. We collected the mothers of experimental females from the wild and allowed them to give birth. The fry were then placed in 3-L aquaria in groups of up to five. From 4 weeks of age onward, these fry were checked weekly for signs of maturation. As soon as we could determine their sex (elongation of the anal fin for males, development of eggs visible through the body wall for females), fish were placed in single sex tanks. Elongation of the anal fin occurs well before males are ready to mate, so we are sure that these fish were virgins at the commencement of the experiment. Virgin females were 3–9 months old when used in our experiment. The use of virgin females ensured that all offspring were sired by males from our experimental pools.

Importantly, prior to placement in experimental pools, both sexes underwent a priming period. This mimicked the experimental conditions that fish would later experience to ensure that paternity results reflected the treatments experienced and not a sudden change from stock to experimental conditions. For priming, focal males and females were placed in experimental pools with the appropriate number of individuals of the opposite sex. Focal males were placed with stock females, and focal females were placed with stock males whose gonopodium tip had been removed to prevent sperm transfer (Mautz, 2011). After 4 days of priming, focal fish were placed directly into their respective experimental treatments, and stock fish were returned to stock tanks.

Once in experimental pools, focal fish had 14 days to interact and mate. They were fed thawed frozen *Artemia* nauplii twice daily. The female-biased treatments were fed twice the amount of food as the male-biased treatments as there were twice as many fish.

Males were euthanized after being removed from the experimental pools. We photographed their left side alongside a microscale using a digital camera (Nikon Coolpix 5700) mounted to a dissecting microscope (Leica Wild MZ8). Males were then preserved in absolute ethanol and stored at −20°C. We later measured male standard length and gonopodium length in IMAGEJ. As male Gambusia have determinate growth (Zulian, Bisazza, & Marin, 1993), measuring their size after the experimental treatment gives an accurate measure of their size during the treatment when competing for mates.

Once females were removed from the experimental pools, they were anaesthetized in ice slurry, photographed and then placed individually in 1-L tanks. Each tank contained a gravel substrate, plastic aquarium plants and a mesh divider to reduce maternal cannibalism. Tanks were checked twice daily for fry until the female had either produced two broods, or 3 months had passed. We collected fry from two broods to increase our sample sizes. Females were kept at 27 ± 1°C on a 14:10 light:dark cycle and fed live *Artemia* twice daily. When a female gave birth, she was placed in a new 1-L tank if it was her first brood. If it was her second brood, she was euthanized and preserved for genotyping. All fry were euthanized (<24 hr after birth) and preserved in family groups of up to 10 fry/vial.

2.3 | Sampling for paternity analysis

To determine male reproductive success, we took tissue samples from up to five mothers (on average 4.1 per pool, $n = 100$ in total), all possible sires (10 per pool, $n = 240$ in total) and all offspring from the selected mothers for each pool (mean: 35.2 per pool, $n = 844$ in total). In the male-biased OSR treatment, we therefore sampled all mothers that gave birth, and in the female-biased OSR treatment, we randomly sampled five females that gave birth. By so doing, we ensured that our power to detect multiple paternity and to detect selection on male traits was similar in the male-biased and female-biased treatments (i.e. in both treatments, we collected data from five females). This sampling

approach did not bias our estimates of selection (see Data S1). DNA was extracted from the tail muscle/caudal fin for adults and from the whole body (excluding head) for fry, using Qiagen DNeasy Blood and Tissue Kits (Qiagen) following the manufacturer's instructions.

After extraction, DNA samples were sent to a commercial genotyping service—Diversity Arrays. The details of the process are described in the Data S1 (see also Booksmythe, Head, Keogh, & Jennions, 2016). We obtained a dataset of *c.* 3,171 SNPs with an average call rate of 97.7% and a reproducibility rate of 99.3%. From the selected SNPs, we calculated a Hamming Distance Matrix of all 1,185 individuals (potential sires, mothers and offspring) to determine paternity. Recent studies show that as few as 30 optimized SNPs are sufficient to differentiate among 100,000 individuals using Hamming Distance values (HDV) (Hu, Liu, Jin, Ropers, & Wienker, 2015). All fry were lined up against their mother and siblings, and the HDVs evaluated to crosscheck for any sample mix ups. None were detected. HDVs were then compared against each of the 10 potential sires. The sire/fry with the lowest value was considered a match. We could assign paternities unambiguously for all 844 fry. Of these, 740 fry were from first broods and 104 from second broods.

2.4 | 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 experiment (F_{het}: Vega Trejo et al., 2017). This is essentially a measure of genome wide heterozygosity. F_{het} is identical to $1 - F_{hom}$ in Bérénos, Ellis, Pilkington and Pemberton (2016); and to H/L in Szulkin, Bierne, and David (2010).

2.5 | Data analysis

2.5.1 | Sexual selection on males

To determine which male traits influenced his reproductive success and whether this varied across socio-environmental contexts, we ran a GLMM. We treated the number of offspring each male sired as the response variable. OSR and habitat complexity were specified as fixed factors. Male standard length (logged), residual gonopodium length (i.e. residuals of the regression of log gonopodium length on log male length) and heterozygosity were included as covariates. Interactions between each of the three male traits and the two experimental factors were included in the model. We did not include interactions between traits, interactions with random effects or higher order interactions as these were not key to the hypotheses being tested and we did not want to over parameterize our models. We treated pool as a random effect to avoid psuedoreplication and specified a Poisson error structure. To account for overdispersion, we included individual as a random effect (Harrison, 2014). Following this correction, our data were underdispersed (dispersion parameter = 0.13) and thus conservative. Although some of the male traits in the model were significantly correlated (see Section 3), collinearity was not a problem for

our model because these correlations were weak. A linear mixed effects model using power-transformed offspring number with the same model structure described above gave qualitatively similar results. Although the residuals from both models looked approximately normal, the linear model provided a worse fit to our data (log-likelihood test: χ^2 = 250.2, *p* < .001) and so the results presented here are from the Poisson model.

To allow comparison between studies, we also calculated the effect sizes for each of the parameters in our model. Effect sizes were calculated using the *p* values from the model following the formulae given in Lipsey and Wilson (2001). By convention, we refer to *r* = .1, .3 and .5 as small, medium and large effect sizes, respectively (Cohen, 1988). To calculate effect sizes, we used *N* = pools (i.e. the number of independent replicates) rather than *N* = males, because the latter potentially underestimates the effect size. The sign of the effect size is based on the direction of the estimate in the model.

Neither the OSR nor habitat complexity influenced the relationship between the number of offspring sired and any of the male traits (see Section 3), so we calculated experiment-wide selection gradients using a linear multiple regression (Lande & Arnold, 1983). We treated the relative number of offspring a male sired (calculated within pools) as the response variable and log male length, residual gonopodium length and heterozygosity as predictor variables. All predictor variables were standardized across the experiment (*M* = 0, *SD* = 1). Standardizing traits within pools gave very similar selection gradient estimates. Significance values were obtained from the same model except that the relative number of offspring sired was power transformed to account for its non-normal distribution and pool identity was treated as a random effect to avoid pseudoreplication. We estimated non-directional selection on each trait using the recently developed method of Henshaw and Zemel (2016), which quantifies the total strength of non-directional selection of any kind (e.g. stabilizing or disruptive selection: Brodie, Moore, & Janzen, 1995). We found no evidence for non-directional selection on any of the three traits (see Data S1 for details).

Incomplete sampling of an individual's mates or offspring can lead to systematic bias in estimates of sexual selection (e.g. the opportunity for sexual selection and the Bateman gradient: Mobley & Jones, 2013; Jones, 2015). It is possible that our subsampling of females in the pools with female-biased OSR might introduce bias in our estimates of selection gradients on male traits (i.e. some males that sired offspring will not be noted as such if the female with whom they mated is not among the five sampled females). To investigate the extent of this problem, we ran simulations to test the effects of subsampling. These simulations demonstrate that any bias is negligible (see Data S1 for details).

2.5.2 | Number of sires per brood

We ran a GLMM with the number of sires per brood as the response variable and OSR, habitat and their interaction as fixed effects. Pool identity was treated as a random effect to avoid pseudoreplication, and we specified a poisson error distribution. This model gave qualitatively similar results to a linear mixed model on transformed data.

As Bolker et al. (2009) does not recommend including random factors with fewer than 5–6 levels, we report results without block as a random effect. Note, however, that including block as a random effect in addition to that of pool did not influence any of our results. All analyses were conducted in R version 3.2.0 (R Core Development Team, 2015). The LME4 package (Bates, Maechler, Bolker, & Walker, 2015) was used to construct models, and p values were obtained using lmerTest.

3 | RESULTS

3.1 | Sexual selection on males

More heterozygous males were smaller (Pearson's correlation: $r = -.164$, $t_{(234)} = 1.354$, $p = .012$) and had a relatively longer gonopodium for their body size (Pearson's correlation: $r = .187$, $t_{(232)} = 2.901$, $p = .004$) (see Figures S2 and S3). Smaller males and more heterozygous males both had significantly greater reproductive success, but there was no effect of the OSR or habitat complexity on sexual selection on any of the three focal male traits (Table 1, Figures S4 and S5). When we examined net selection across all four treatments, the selection gradients were statistically significant for all three male traits (Table 2). It is worth noting that the patterns are very clear: in 22 of 24 pools selection favoured more heterozygous males; in 19 of 24 pools selection favoured smaller males; and in 20 of 24 pools selection favoured males with a relatively long gonopodium.

3.2 | Number of sires per brood

The mean number of sires per brood was greater under a male-biased than female-biased OSR (estimate ± SE = 0.420 ± 0.192,

TABLE 2 The vector of experiment-wide standardized linear selection gradients (β) for male traits in *Gambusia holbrooki*. Relative fitness was calculated within pools, and male traits were standardized across the experiment. Selection gradients were estimated using linear multiple regression. The significance of selection gradients was determined using a linear mixed model with power-transformed relative fitness as the response variable to account for non-normal distribution of the data. Pool was included in this model as a random effect to account for potential non-independence of data from the same pool

Trait	B (SE)	p
Per cent heterozygosity	0.355 (0.103)	**<.001**
Log standard length	−0.205 (0.101)	**<.001**
Residual gonopodium length	0.348 (0.101)	**<.001**

Bold indicates significant effect.

$Z = 2.181$, $p = .029$), but it did not depend on habitat complexity (estimate ± SE = −0.095 ± 0.195, $Z = 0.488$, $p = .626$), nor was there an interaction between OSR and habitat complexity (estimate ± SE = −0.125 ± 0.280, $Z = 0.446$, $p = .655$) (Figure 1). This finding is unlikely to be confounded by female fecundity depending on the OSR (see Data S1).

4 | DISCUSSION

Spatial and temporal environmental and/or social heterogeneity have long been invoked as factors that help to maintain variation in traits that are under directional sexual selection (Cornwallis & Uller, 2010; Levins, 1968). We experimentally tested how two key

TABLE 1 The effects of operational sex ratio (OSR) and habitat complexity on the relationship between male traits and the number of offspring sired. Effect sizes (r) and their 95% confidence intervals were calculated from p following the formula in Lipsey and Wilson (2001)
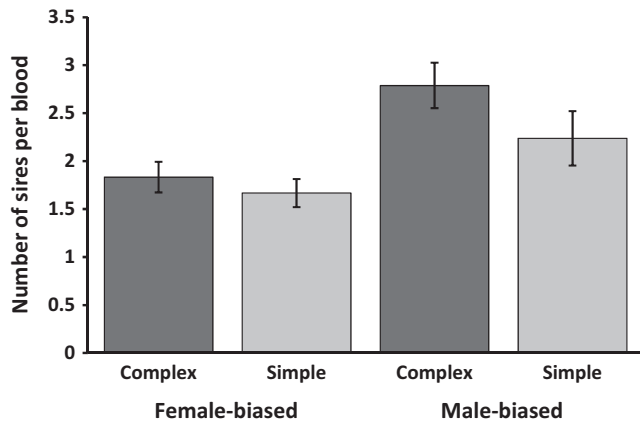
Trait	Term	Estimate	SE	z	p	r
Number of offspring	Intercept	16.139	12.603	1.370	.181	
	OSR (m)	−2.790	15.927	−0.191	.861	.038
	Habitat (s)	0.301	16.600	0.016	.986	.004
	Log standard length (SL)	−18.414	8.034	−2.335	**.022**	.465
	Residual gonopodium length (Gono)	0.479	0.331	1.420	.148	.305
	Per cent heterozygosity (Het)	31.336	10.341	3.072	**.002**	.599
	OSR(m) × Habitat (s)	2.131	21.874	0.092	.922	.021
	OSR(m) × SL	1.091	10.576	0.116	.918	.022
	OSR(m) × Gono	−0.395	0.507	−0.704	.436	.167
	OSR(m) × Het	4.456	14.971	0.308	.766	.064
	Habitat(s) × SL	2.007	11.078	0.190	.856	.039
	Habitat(s) × Gono	0.655	0.503	1.346	.193	.275
	Habitat(s) × Het	−11.115	14.159	−0.811	.432	.168
	OSR(m) × Habitat(s) × SL	2.965	14.596	0.210	.839	.044
	OSR(m) × Habitat(s) × Gono	0.305	0.752	0.333	.685	.087
	OSR(m) × Habitat(s) × Het	−21.459	19.683	−1.092	.276	.232

Bold indicates significant effects.

FIGURE 1 The mean number of sires contributing to each brood (±*SE*) within each treatment. The number of females in each treatment: female-biased/complex habitat (*N* = 30); female-biased/simple habitat (*N* = 30); male-biased/complex habitat (*N* = 19); male-biased/simple habitat (*N* = 22)

parameters—the OSR and habitat complexity—influence sexual selection on male mosquitofish *G. holbrooki*. Our estimates of sexual selection on focal male traits did not differ across environments. Small males, males with a relatively larger gonopodium and more heterozygous males had greater reproductive success in all cases. This trend was remarkably consistent in direction across pools (19–22 of the 24 pools). Our results demonstrate that altering OSR and habitat complexity has little effect on sexual selection in *G. holbrooki*.

4.1 | The operational sex ratio

Male-biased sex ratios increase competition for mates, and it is usually assumed that this will increase variation in male mating and reproductive success (Emlen & Oring, 1977; Shuster, 2016). Instead, we found that the mean number of sires per brood was greater with a more male-biased sex ratio even though the number of males in our different sex ratio treatments remained constant. This could indicate that males are better able to monopolize females in female-biased environments. We cannot determine from our experiment whether this effect is due to changes in male or female density, however, because we deliberately kept the overall population density constant (meaning that the density of a given sex and the sex ratio covary perfectly). All else being equal, a greater sharing of paternity might be expected to reduce selection on sexual traits because it reduces variation in male reproductive success. However, the adult sex ratio had no detectable effect on our estimated selection gradients. This reveals that even though more sires contributed to each brood this did not affect the net distribution of paternity among males.

There was no evidence that the sex ratio, as manipulated here, influenced selection on male traits in *G. holbrooki*. Numerous studies have shown that the adult sex ratio and the OSR can both affect mating behaviour (e.g. Bretman, Westmancoat, Gage, & Chapman,

2012; Holveck, Gauthier, & Nieberding, 2015). However, few studies experimentally manipulate the OSR to test whether it affects sexual selection on specific male traits (most studies simply show that the OSR affects male trait expression: see Weir et al., 2011). This is a surprising oversight as the evolution of male traits depends on how they affect fitness (i.e. relative number of offspring sired): for the OSR to affect evolution it must alter the trait–fitness relationship. Of the experimental OSR studies that measure selection on male traits based on actual reproductive success, the results are mixed. In guppies (*Poecilia reticulata*, Head et al., 2008) and bank voles (*Clethrionomys glareolus*, Mills, Grapputo, Koskela, & Mappes, 2007), there was no effect of the OSR on selection on male sexual traits. In contrast, in two-spotted gobies (*Gobiusculus flavescensa*, Wacker et al., 2013) and rough-skinned newts (*Taricha granulosa*, Jones, Arguello, & Arnold, 2004), selection on male traits was stronger when the OSR was more male-biased. Finally, and contrary to some expectations, selection on male traits was weaker when the OSR was more male-biased in bank voles (*Clethrionomys glareolus*, Klemme, Yloenen, & Eccard, 2007) and common lizards (*Lacerta vivipara*, Fitze & Le Galliard, 2011).

The conflicting results in previous studies of how the OSR affects sexual selection might be partly due to confounding effects of other ecological parameters, especially those that determine how often individuals interact (e.g. habitat complexity, or factors that influence population density [see Kokko & Rankin, 2006]). We therefore tested for an interaction between the OSR and habitat complexity that might affect sexual selection in *G. holbrooki*. There was no evidence that habitat complexity, at least as manipulated in our study, affected sexual selection on the three measured traits, either by moderating the effect of the OSR or by having a consistent effect irrespective of the OSR. Another environmental parameter, that we did not manipulate in our experiment, that may be important in mediating the effects of OSR on sexual selection is population density (but see Head et al., 2008; Wacker et al., 2013). In our experiment, fish densities were at the high end of what fish might experience in the wild, and thus, it is possible that consistent sexual selection for small males with a long gonopodium (two traits that might be expected to be favoured when there is more potential for sexual coercion) is the result of high densities across all of our treatments. This hypothesis remains to be tested.

4.2 | Habitat complexity and sexual selection

Habitat complexity, as manipulated here, did not influence selection on male traits, even though comparable variation in habitat complexity sometimes alters sexual behaviour in other poeciliid fishes (e.g. Hibler & Houde, 2006). Furthermore, habitat variation is important in shaping sexual traits in many species, which is why ecological factors are often implicated in population variation in sexual traits (e.g. Cornwallis & Uller, 2010) and even in speciation (e.g. Maan & Seehausen, 2011). For example, habitat differences in gravel size promote divergence in male coloration in guppies (*Poecilia reticulata*, Endler, 1980). Similarly, variation in habitat complexity affects selection on male advertisement calls in cricket

frogs (*Acris crepitans*, Ryan, Cocroft, & Wilczynski, 1990). The difference between these studies and ours might reflect the relationship between the traits being measured and the environment they are being measured in. In the studies mentioned above, coloration and vocalizations are sexual signals whose transmission and detection is dependent upon the habitats they are being measured in. In contrast, in our study body size, residual gonopodium size and heterozygosity are not. In our study, a more likely mechanism by which habitat complexity would alter selection is via effects on mate encounter rates, which may affect how females assess males, or might shift the balance between different modes of sexual selection (e.g. mate choice vs. coercion).

4.3 | Traits under sexual selection in *G. holbrooki*

We detected strong directional selection on males for smaller body size, larger residual gonopodium length and higher heterozygosity. We consider each trait in turn.

4.3.1 | Male body size

Smaller male *G. holbrooki* had greater reproductive success. This has long been assumed for *Gambusia* spp. based on indirect behavioural evidence for insemination success (e.g. Pilastro et al., 1997), but until recently paternity data have been lacking. In a small paternity study, Deaton (2008) found a large male advantage based on 27 trials where a small and a large male competed freely for access to a female within small aquaria. In a much larger study of 180 males, Booksmythe et al. (2016) found no effect of male body size on paternity in 30 pools, in each of which six males freely competed for eight females. As in many species of poeciliid fishes, male mosquitofish vary substantially in size (range in this experiment: 19–32 mm). Understanding how this type of variation persists despite strong directional selection is a major challenge (Barton & Turelli, 1989). This is true even if there is no additive genetic variation in male size (but see Stearns, 1983). Potential explanations include context-dependent selection (Cornwallis & Uller, 2010), genic capture (Tomkins, Radwan, Kotiaho, & Tregenza, 2004), fitness trade-offs between traits (Blows, Brooks, & Kraft, 2003) and trade-offs between the effect of a given trait under different modes of selection (Devigili et al., 2015; Johnston et al., 2013). Our current results, while they clearly need to be replicated in a wider range of habitats, suggest that large size variation in male *G. holbrooki* is not due to variation in habitat complexity, nor to ecological factors that affect the OSR.

4.3.2 | Male genital size

Male *G. holbrooki* with a relatively long gonopodium for their body size had higher reproductive success. Similar positive directional selection on gonopodium length has been shown previously in guppies, *P. reticulata* (Devigili et al., 2015; Evans et al., 2011), and in a second study on *G. holbrooki* from our laboratory (Vega Trejo et al., 2017).

This could be due to female choice for males with a long gonopodium (Kahn et al., 2010; Langerhans, Layman, & DeWitt, 2005), or a greater ability to inseminate females coercively (Evans et al., 2011). Interestingly, Booksmythe et al. (2016) recently showed no increase in reproductive success for males from lines artificially selected for greater residual gonopodium length. This suggests that although residual gonopodium length is heritable (i.e. it evolved under artificial selection) and there is also directional *selection for* males with a relatively long gonopodium for their body size, this might not be due to *selection of* a relatively long gonopodium (see Morrissey (2014) for a discussion on the distinction between "selection for" and "selection of" a trait). That is, an unmeasured factor might cause both greater residual gonopodium length and higher reproductive success. A likely candidate is body condition (see also Kruuk et al., 2002). Alternatively, the evolution of gonopodium length might be constrained by genetic covariance with other traits that affect fitness, so that the response to artificial selection is largely orthogonal to the unmanipulated direction of selection in multivariate trait space (Blows, Chenoweth, & Hine, 2004; Hine, Chenoweth, & Blows, 2004). This is a reminder of the easily overlooked fact that estimates of selection gradients can only truly estimate direct selection on traits if all relevant covarying traits are measured (Lande & Arnold, 1983).

4.3.3 | Heterozygosity

Male *G. holbrooki* with higher heterozygosity had greater reproductive success. This is a finding that we have recently replicated in a second paternity analysis study after using a formal breeding design to systematically manipulate male heterozygosity (Vega Trejo et al., 2017). Studies of heterozygosity fitness correlations (HFCs) show that homozygosity negatively affects fitness-enhancing traits (reviews: Chapman, Nakagawa, Coltman, Slate, & Sheldon, 2009; Coltman & Slate, 2003; Szulkin et al., 2010). There are, however, relatively few HFC studies that link heterozygosity to male reproductive success under sexual selection (i.e. control for male mortality). Of these, several studies show that lower heterozygosity decreases male reproductive success (e.g. water dragons, *Intellagama lesueurii* [Frere, Chandrasoma, & Whiting, 2015]; Black rhinoceros, *Diceros bicornis michaeli* [Cain et al., 2014]; zebra finches, *Taeniopygia guttata* [Forstmeier, Schielzeth, Mueller, Ellegren, & Kempenaers, 2012]; house mice, *Mus musculus musculus* [Thoss, Ilmonen, Musolf, & Penn, 2011]; blue tits, *Cyanistes caeruleus* [Olano-Marin, Mueller, & Kempenaers, 2011]), although this is not always true (e.g. Great tits, *Parus major* [Chapman & Sheldon, 2011]).

We observed a strong positive relationship between heterozygosity and male reproductive success ($r = .267$) compared to a mean value for HFCs of $r = .05$ (meta-analysis: Chapman et al., 2009). There are several reasons why we might see a strong relationship in *G. holbrooki*. First, we had a better estimate of genome-wide heterozygosity (Balloux, Amos, & Coulson, 2004). Although microsatellite markers are generally 4–10 times more variable than SNPs (Mariette, Le Corre, Austerlitz, & Kremer, 2002; Morin, Luikart, Wayne, & Grp, 2004), the 3,171 SNP markers we used is equivalent

to using over 300 microsatellite markers. To date, most HFC studies use <20 microsatellite markers (Chapman et al., 2009). Secondly, traits that are more closely related to actual fitness are more likely to suffer inbreeding depression (Kristensen, Pedersen, Vermeulen, & Loeschcke, 2010). The studies in Chapman et al. (2009) mainly report HFC correlations for morphological, physiological and life-history traits; very few studies provide direct fitness estimates such as reproductive success. As such, the average HFC in Chapman et al. (2009) is likely to be an underestimate of the true link with fitness (Chapman & Sheldon, 2011). Thirdly, the HFC is likely to depend on a population's demographic history, with relationships being weaker in highly outbred or inbred populations where variation in heterozygosity is lower. In our study population, there is a relatively low mean heterozygosity, but it is within the range of natural populations (Vera, Diez-del-Molino, & Garcia-Marin, 2016). Crucially, however, there is substantial variation in heterozygosity (17%–36%), which makes the population particularly conducive to quantifying the HFC. Interestingly, another recent study that used a large number of SNPs to estimate inbreeding also found strong inbreeding depression when looking at fitness traits in red deer (*Cervus elaphus*) (including lifetime reproductive success) despite relatively low variation in heterozygosity (Huisman, Kruuk, Ellis, Clutton-Brock, & Pemberton, 2016).

There are many mechanisms whereby lower heterozygosity could reduce fitness due to negative effects on the ability to acquire mates and fertilizations. Previous studies have shown that inbred (i.e. more homozygous) males can be less attractive (review: Pusey & Wolf, 1996); produce less competitive ejaculates (e.g. Michalczyk, Martin, Millard, Emerson, & Gage, 2010); and have poorer locomotion performance (e.g. Manenti et al., 2015) or poorer cognition (Fareed & Afzal, 2014) that could reduce the ability to locate females. Regardless of the proximate mechanism, however, the reported strong effect of heterozygosity on paternity is likely to have wider implications. For example, by changing the effective population size and identity of successful males, it might affect the persistence and recovery of small populations (Keller & Waller, 2002) (i.e. fewer sires reduce population genetic diversity, but their being more genetically diverse increases population genetic diversity).

5 | CONCLUSION

Sexual selection in the mosquitofish was consistent across populations that differed in two parameters that are expected to affect mate encounter rates. Persistent ecological differences between habitats are clearly important for generating divergence in sexual traits between species (reviewed in Maan & Seehausen, 2011). However, the extent to which temporal and spatial habitat variation generates variation among extant populations of a single species is less clear (Cornwallis & Uller, 2010). Experimental studies like ours that quantify sexual selection in different environments and over different time/spatial scales are needed to understand better how ecological variation affects the strength and form of sexual selection and, by extension, how organisms might respond to changing environments.

AUTHOR CONTRIBUTIONS

M.L.H., A.T.K., J.S.K. and M.D.J. designed the experiment; M.L.H. and A.T.K. collected the data; M.L.H., J.M.H., J.S.K. and M.D.J. analysed the data; M.L.H. wrote the first draft of the manuscript and all authors helped with revisions.

DATA ACCESSIBILITY

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.93351 (Head, Kahn, Henshaw, Keogh, & Jennions, 2017).

ORCID

Megan L. Head [ID] http://orcid.org/0000-0002-8123-7661

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