

Shifts in Reproductive Investment in Response to Competitors Lower Male Reproductive Success

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ABSTRACT: In many species, males exhibit phenotypic plasticity in sexually selected traits when exposed to social cues about the intensity of sexual competition. To date, however, few studies have tested how this plasticity affects male reproductive success. We initially tested whether male mosquitofish, *Gambusia holbrooki* (Poeciliidae), change their investment in traits under pre- and postcopulatory sexual selection depending on the social environment. For a full spermatogenesis cycle, focal males were exposed to visual and chemical cues of rivals that were either present (competitive treatment) or absent (control). Males from the competitive treatment had significantly slower-swimming sperm but did not differ in sperm count from control males. When two males competed for a female, competitive treatment males also made significantly fewer copulation attempts and courtship displays than control males. Further, paternity analysis of 708 offspring from 148 potential sires, testing whether these changes in reproductive traits affected male reproductive success, showed that males previously exposed to cues about the presence of rivals sired significantly fewer offspring when competing with a control male. We discuss several possible explanations for these unusual findings.

Keywords: reproductive success, sexual selection, precopulatory traits, postcopulatory traits, sperm competition, ejaculate.

Introduction

Males face the dual challenge of acquiring mates and fertilizing eggs (Evans and Garcia-Gonzalez 2016). Precopulatory sexual selection favors male traits, such as sexual ornaments, courtship, weapons, and fighting ability (Kokko et al. 2006; Emlen 2008; Kuijper et al. 2012; Hardy and Briffa 2013). Postcopulatory sexual selection favors male traits that increase fertilization success under sperm competi-

tion, such as ejaculate size (Parker and Pizzari 2010; Kelly and Jennions 2011) and sperm morphology (Lüpold et al. 2009, 2016; Firman and Simmons 2010). The optimal allocation to different sexually selected traits depends on investing until the marginal fitness gains per trait are identical (Simmons et al. 2017; Hooper et al. 2018; Fox et al. 2019). This can select for adaptive phenotypic plasticity if gains vary predictably across environments (Punzalan et al. 2010). In principle, males can use cues in their current environment to predict future levels of sexual competition. They can then adjust their allocation to different traits based on the relative marginal gain each is likely to provide (review: Dore et al. 2018; Fox et al. 2019).

Evidence of sexual trait plasticity comes from experiments that manipulate social cues about sexual competition, such as sex ratio or male density (e.g., Cattelan et al. 2016; Rouse et al. 2018; Sloan et al. 2018; Hopkins et al. 2019; review: Bretman et al. 2011a). Given greater mating competition, males should generally allocate more to traits that improve their ability to compete for and gain mates. For instance, male house sparrows (*Passer domesticus*) in dense colonies increase their mating effort (Hoi et al. 2011), and male Siamese fighting fish (*Betta splendens*) court more vigorously when a rival is present (Dzieweczynski et al. 2009). Similarly, males should also allocate more to traits under postcopulatory sexual selection when sperm competition is elevated (Parker and Pizzari 2010). Theoretical models predict that greater sperm production is selected for when the risk or intensity of sperm competition rises (Williams et al. 2005; Fromhage et al. 2008; Tazzyman et al. 2009; Parker et al. 2013). For example, male house mice (*Mus musculus domesticus*) that encounter the scent markings of rivals more frequently increase their daily sperm production (Ramm and Stockley 2009). Many experimental studies have shown male phenotypic plasticity in both pre- and postcopulatory sexually selected traits in response to manipulation of social cues (meta-analysis:

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Kelly and Jennions 2011; Weir et al. 2011; review: Bretman et al. 2011a). But what is the optimal investment strategy in response to social cues indicative of future sexual competition? Investment into mate acquisition reduces the resources available for competitive ejaculates, and vice versa (review: Simmons et al. 2017). It is therefore difficult to predict the extent to which selection favors a shift in allocation to pre- and postcopulatory traits. Changes in the social environment tend to simultaneously alter the level of both mating and sperm competition (Parker et al. 2013; Órfão et al. 2018). To further complicate matters, the social environment can alter natural selection on traits (e.g., population density affects feeding competition), and changes in body condition affect the optimal allocation strategy (Tazzyman et al. 2009; Hooper et al. 2018; Fox et al. 2019).

Ultimately, we need to quantify male reproductive success to test how plasticity in sexual traits following exposure to social cues affects fitness. First, because of trade-offs, we have to investigate the net effect of shifts in traits that determine fertilization and mating success. Second, male reproductive success can be affected even if there are no measurable changes in key traits that predict fertilization or mating success. For example, inbred males gain less paternity in eastern mosquitofish (*Gambusia holbrooki*), despite no detectable inbreeding depression for sperm traits (Marsh et al. 2017; Vega-Trejo et al. 2017). Third, female control of mating and fertilization could oppose shifts in allocation that should otherwise increase male success (Snook 2005; Bretman et al. 2011a). These three considerations mean that we have to quantify the relative reproductive success of males exposed to cues that either match or mismatch the future competitive environment when they compete freely for mates and fertilizations. To date, few studies have pursued this approach. The first such study showed that male fruit flies (*Drosophila melanogaster*) adjust their ejaculates in response to manipulation of cues about the level of sperm competition and confirmed that this increases their reproductive success (Bretman et al. 2009a). Later studies on other insects provided further evidence for similar adaptive plasticity in *D. melanogaster* (Wigby et al. 2009; Bretman et al. 2011b, 2013; Hopkins et al. 2019), *Drosophila pseudoobscura* (Price et al. 2012), and soldier flies (*Merosargus cingulatus*; Barbosa 2012), but the applicability of these results to other taxa, especially vertebrates, is unknown.

Here we used the eastern mosquitofish, *G. holbrooki*, to test whether exposure to social cues presumed to indicate the level of sexual competition affects male investment in different sexually selected traits and ultimately reproductive success. We experimentally manipulated the perceived level of sexual competition by placing males in either a competitive or a control environment for a full spermatogenesis cycle. More specifically, we provided test males with visual and olfactory access to a female that was either

alone or housed with rival males. We then investigated whether this affected male behavior and/or sperm production. Crucially, we then tested for an effect of the social treatment on male reproductive success when males from each treatment competed one-on-one with each other to sire offspring. We predicted that males that were previously exposed to rivals—indicative of greater mating and sperm competition—would (a) increase their mating effort and/or sperm production and (b) sire more offspring.

Methods

Study Species

Gambusia holbrooki naturally occur in highly variable social environments (Pyke 2005; Kahn et al. 2013). Male mating behavior is almost exclusively confined to gonopodial thrusting, where a male approaches the female from behind, swings his gonopodium (an intromittent organ modified from the anal fin) forward, and tries to insert it into her gonopore (Bisazza 1993; see video 1, available online). Males continually pursue females, with males in some populations attempting on average one mating per minute (Bisazza and Marin 1995; Wilson 2005). However, males also perform courtship displays, albeit at a much lower frequency than other poeciliids (Peden 1970; Wang et al. 2015). During these displays, the male is initially motionless for a few seconds in front of the female, while the pigments on his lower jaw and lateral stripe quickly darken, and he then swims rapidly to display his lateral fins (Peden 1970; see video 1).

Mosquitofish have internal fertilization, and males transfer sperm (packaged in bundles) using their gonopodium. Females can store sperm for several months (Constantz 1989) and have broods with multiple paternity (Zane et al. 1999; Booksmythe et al. 2016). At low densities, while larger, socially dominant males chase rivals away from a female (Bisazza 1993; see video 1), successful insemination actually depends on the male-to-female size ratio, with smaller males being favored (Bisazza and Marin 1995; Pilastro et al. 1997; Head et al. 2017; but see Booksmythe et al. 2016; Vega-Trejo et al. 2017). As in most poeciliids, adult females are larger than adult males (Bisazza 1993).

Origin and Maintenance of Fish

Experimental males were collected from natural ponds in Canberra, Australia, in February–March 2016 and kept in the laboratory for at least 1 week before the start of the experiment. We used wild-caught males, because we felt it was more important to use males that had initially experienced natural social conditions than to use lab-reared males, even though this would have allowed for better control of their past history. Experimental females were the



Video 1: Still photograph from a video showing mating behaviors.

daughters of wild-caught females. All test fish were housed in single-sex aquaria (30–60 fish per 90 L) to ensure female virginity and to prevent males from using their sperm reserves. Fish were maintained under a 14L:10D photoperiod at 28°C and fed with *Artemia salina* nauplii and fish flakes twice a day.

Experimental Design: Social Treatment

We manipulated social cues about reproductive competition. The experimental protocol is summarized in figure 1.

We created 83 size-matched male pairs (mean difference: 0.036 ± 0.010 mm) to eliminate any initial difference in social dominance (see Harrison et al. 2018). There is minimal postmaturation male growth (<0.06 mm/week; Iglesias-Carrasco et al. 2019). Males were anesthetized in icy water (~ 10 s), placed on a black background in a petri dish alongside a microscopic ruler (0.1-mm gradation), and photographed. We measured standard length (SL; snout tip to base of caudal fin) and gonopodium length using the program ImageJ (Abràmoff et al. 2004). Males were individually marked with a small dot of colored fluorescent elastomer

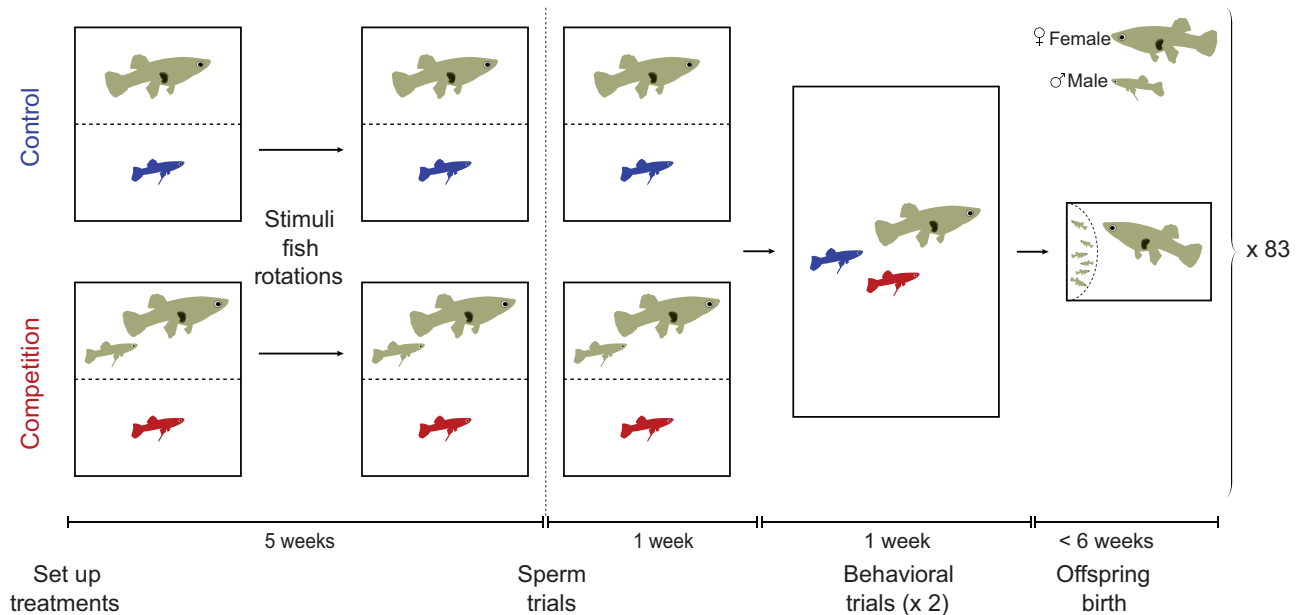


Figure 1: Experimental protocol for the 83 size-matched male pairs. The dashed vertical line indicates when males were stripped of sperm, the dashed horizontal lines represent mesh barriers within the treatment aquaria, and the dashed curved line represents mesh within the offspring birth aquaria. Blue indicates control males, and red indicates males exposed to the competition treatment.

(Northwest Marine Technology, Anacortes, WA), which was injected subcutaneously behind the caudal fin, and had at least a day to recover before being placed in a social treatment.

For each pair, we randomly assigned one male to the control and the other to the competition treatment. By size-matching males we eliminated any difference in dominance because of size, which is the key predictor of social dominance (Harrison et al. 2018). In both cases, we used a 7-L aquarium divided in half by a mesh barrier. We placed a focal male in one compartment, and in the other we placed either a stimulus female (control) or a stimulus female with a rival male (competition treatment). All stimulus females and rival males were taken at random from stock tanks and rotated weekly to maintain the focal males' sexual interest. The mesh barrier provided the focal male with visual and olfactory cues of rivals but prevented physical contact. We fed fish twice daily, ensuring that the food per capita was equal.

Males were exposed to the social treatments for 5 weeks, which exceeds the length of a full spermatogenesis cycle (Koya and Iwase 2004). We then stripped males of sperm to quantify sperm number and velocity. To restore their sperm reserves, males were returned to their treatment aquarium for another week (O'Dea et al. 2014). Next, we placed the pair of size-matched males with a virgin female in a 90-L aquarium. We recorded their mating behavior on the first and third day (see below). After 1 week, males were euthanized and stored in 100% ethanol for paternity analysis. Females were transferred to 1-L aquaria with a mesh-partitioned compartment to protect offspring from maternal cannibalism. We checked the aquaria twice daily for offspring and fed females ad lib. twice daily until they gave birth. Females were then photographed and measured.

Sperm Number and Velocity

We collected sperm from 161 males (five died before sampling). Each male was anesthetized in icy water and then placed on a glass slide coated with 1% polyvinyl alcohol (PVA) to prevent sperm bundles from sticking to the slide. Under a dissecting microscope, we swung the gonopodium forward and applied gentle pressure to the abdomen to eject the available sperm. The mean number of bundles per stripped male is >800 (R. J. Fox, E. E. Gearing, M. D. Jennions, and M. L. Head, unpublished data). Six sperm bundles were used for sperm velocity analyses. Using a pipette, we transferred the remainder to an Eppendorf tube with 100–900 μL of extender medium (pH 7.5 with composition 207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl_2 , 0.49 mM MgCl_2 , 0.41 mM MgSO_4 , 10 mM Tris), depending on the amount of ejaculate stripped. Sperm are quiescent in this solution (Gardiner 1978). Afterward, we

returned males to their treatment tanks for another week until the behavioral trials started. Sperm collection was done blind to treatment type by R. Vega-Trejo.

To estimate the number of sperm, we vortexed the sperm solution for 1 min and mixed it with a pipette (20–30 times) to break the sperm bundles and distribute the sperm evenly throughout the sample. We then placed 3 μL on a 20- μm capillary slide (Leja) and counted sperm using a CEROS sperm tracker (Hamilton Thorne, Beverly, MA) under $\times 100$ magnification. The threshold values defining cell detection were set as elongation percentage 15–65 and head size 5–15 μm , and the static tail filter was off. We counted five subsamples and estimated repeatability using the rptR package (Nakagawa and Schielzeth 2010). Repeatability was high ($r = 0.84 \pm 0.02$ [SE], $P < .001$), and the mean sample value was used in further analyses. The sperm count was corrected for the six bundles used to estimate sperm velocity. The mean number of sperm per bundle does not vary significantly among males ($7,677 \pm 477$ [SE], $n = 50$ males; R. J. Fox, E. E. Gearing, M. D. Jennions, and M. L. Head, unpublished data).

To measure sperm velocity, we created two samples per ejaculate consisting of three bundles in 2 μL of extender medium. Each sample was placed in the center of a cell of a 12-cell multitest slide (MP Biomedicals, Irvine, CA) coated with 1% PVA solution to prevent sperm from sticking to the slide. The sperm were then activated with 4 μL of solution (150 mM KCl and 2 mg/mL bovine serum albumin; Billard and Cosson 1992) and covered with a coverslip. Within 30 s of activation, we estimated sperm velocity on the basis of 26.0 ± 1.3 (SE) sperm tracks per ejaculate (with a minimum of 10 tracks/male). We recorded two standard measures of sperm velocity: (i) velocity over a smoothed average path (VAP) and (ii) curvilinear velocity (VCL), the average velocity along its actual trajectory. The threshold values defining static cells were set at 20 $\mu\text{m}/\text{s}$ for VAP and 15 $\mu\text{m}/\text{s}$ for VCL. Given the significant repeatability of VAP and VCL (VAP: $r = 0.31 \pm 0.09$ [SE], $P < .001$; VCL: $r = 0.27 \pm 0.09$, $P < .001$), we used the mean value in our analyses. VAP and VCL are highly correlated ($r = 0.91$, $P < .001$), so we used VCL in our analyses ($N = 132$ males). Using VAP produced nearly identical results.

Mating Behaviors

One week after sperm collection (total social treatment of 6 weeks), we observed male reproductive behavior in mating trials. First, we allowed the pair of size-matched competition treatment and control males and a virgin female to acclimate in a 90-L tank for 5 min. We then recorded all copulation attempts and courtship displays performed by each male for 10 min on the first and the

third day. The behavioral data from 76 of 83 available pairs ($N = 152$ sessions—in seven pairs, one of the males died) were collected blind to treatment type and fish identity by F. Spagopoulou, using the program JWatcher (Blumstein et al. 2000). Copulation attempts and courtship displays occur only if a male is near a female. Therefore, these behaviors partly reflect the extent to which a male gains greater access to a female by chasing away his rival and summarize male-male aggressiveness and social dominance (see “Discussion”).

Paternity Analysis

To assign paternity, we single-nucleotide polymorphism (SNP) genotyped up to 10 offspring per female that bred ($N = 74$ females, mean = 9.57 offspring, total $N = 708$ offspring and 148 males genotyped; mean brood size was 19.97 offspring). Using the data from our initial 83 pairs, the power to detect a medium-strength effect of social treatment sensu Cohen (1988) is 80% with a sign test (i.e., which male per pair sired more offspring). We genotyped fish using the commercial services of Diversity Arrays, who use DArTseq (see the supplemental PDF, available online). We could unambiguously assign the paternity of all offspring.

Statistical Analyses

All statistical analyses were performed in R version 3.3.1 (R Development Core Team 2017). Statistical models were fitted with the lme4 package (ver. 1.1-12). For general linear models (LMs), we ensured an appropriate fit by evaluating the distribution of model residuals. For generalized linear mixed models (GLMMs; Poisson or binomial error structure), we ensured an appropriate fit using the DHARMA package (ver. 0.1.2) for diagnostic tests of model residuals (Hartig 2016). To test the significance of model terms, we used the Anova function of the car package (ver. 2.1-3), with type III Wald χ^2 tests. The P values obtained from the Anova summaries and the parameter estimate summaries were similar (as categorical variables had only two levels). We report the significance of the parameter estimates, because in some cases we were also interested in the intercept. All data and the R script used for the analyses have been deposited in the Dryad Digital Repository (<https://doi.org/10.5061/dryad.v41ns1rs9>; Spagopoulou et al. 2020).

We analyzed behaviors on days 1 and 3 separately, and the results were broadly congruent (table 1). We constructed separate generalized linear models (GLMs) for copulation attempts and courtship displays, with treatment type as a fixed factor and standardized male size (SL) as a fixed covariate. We tested whether the treatment type and size determined (i) whether a male performed

the behavior in question (GLM with binomial error structure) and (ii) the rate for males that showed the behavior (GLMM with Poisson error structure and random factors: pair ID and an observation-level random effect to control for overdispersion; Harrison 2014). The treatment-by-size interaction was not significant. Therefore, it was removed from all final models, except the binomial models for day 3.

To examine the effect of the social environment on sperm number and velocity, we performed separate LMs (table 2). We square root transformed sperm number to fulfil normality assumptions. In all models, the treatment type was treated as a fixed factor and standardized male size (SL; Schielzeth 2010) as a fixed covariate. The treatment-by-size interaction was not significant and was thus removed from the final model.

Finally, to test whether the social competition treatment affected male reproductive success, we constructed a GLM for the proportion of offspring sired by the competition treatment males (weighted by the number of offspring genotyped using the cbind function), using a quasi-binomial error structure to control for overdispersion (table 3). We included the differences between males (i.e., competition treatment – control) in size (SL), relative gonopodium length (i.e., residuals from log gonopodium on log SL regression), and heterozygosity (i.e., proportion of heterozygous SNP loci) as fixed covariates. These traits have predicted paternity in previous studies of *G. holbrooki* (Bookmythe et al. 2016; Head et al. 2017; Marsh et al. 2017; Vega-Trejo et al. 2017). We also included female size as a covariate in case the social treatment induced differential investment on the basis of female size (Edward and Chapman 2011). All covariates were standardized (mean = 0, SD = 1). A significant deviation from a zero intercept reveals a social treatment effect on paternity, when mating with an average-sized female and there is no male difference in the measured traits. In this analysis, we excluded two pairs with high leverage (for an analysis with these pairs included, see table S1, available online).

Results

Mating Behavior

On day 1, 148 out of 152 males (97%) attempted to copulate. The competition treatment did not affect whether males tried to copulate ($Z = 0.0$, $P = 1.000$). On day 3, 141 out of 152 males (93%) tried to copulate, and there was a significant interaction between treatment and male size ($Z = -2.56$, $P = .010$). Among competition treatment males, larger individuals were less likely to initiate copulation attempts, but among control males the opposite was observed. Of the males that initiated copulation

Table 1: Effects of competition treatment and male size on the occurrence and rate of male mating behaviors: copulation attempts and courtship displays

	Copulation attempts (yes/no)		Copulation attempts (rate)		Courtship displays (yes/no)		Courtship displays (rate)	
	Estimate ± SE	Variance	Estimate ± SE	Variance	Estimate ± SE	Variance	Estimate ± SE	Variance
Day 1:								
Intercept	3.61 ± .51***		3.12 ± .10***		-2.39 ± .29***		.34 ± .25	
Competition (present)	-.00 ± .51		-.13 ± .04***		.08 ± .29		.19 ± .24	
Male size (SL standardized)	.09 ± .52		-.11 ± .09		-.18 ± .31		-.01 ± .47	
Pair ID (random effect)	62	00
Observation (random effect)	14	00
Day 3:								
Intercept	3.15 ± .53***		2.19 ± .07***		.22 ± .17		1.12 ± .08***	
Competition (present)	.79 ± .53		-.22 ± .06***		-.11 ± .17		-.22 ± .07**	
Male size (SL standardized)	-.18 ± .38		.02 ± .06		-.02 ± .17		-.03 ± .07	
Male size × competition (high)	-.99 ± .38**		...		-.36 ± .17*		...	
Pair ID (random effect)	08	04
Observation (random effect)	33	05

Note: Model details are given in the main text. Occurrence of each behavior is the binomial response as “yes” or “no.” Rate of each behavior is the number of occurrences in 10 min. SL = standard length.

* $P < .05$.

** $P < .01$.

*** $P < .001$.

Table 2: Effects of competition treatment and male size on sperm traits: sperm number and sperm velocity

	Sperm no. (estimate \pm SE)	Sperm velocity (VCL; estimate \pm SE)
Intercept	3,024.92 \pm 101.00***	81.71 \pm 1.20***
Competition (present)	34.34 \pm 101.00	-2.46 \pm 1.20*
Male size (SL standardized)	135.56 \pm 101.67	3.32 \pm 1.18**

Note: Model details are given in the main text. Sperm number was square root transformed in the model. Sperm velocity was measured as the curvilinear velocity (VCL). SL = standard length.

* $P < .05$.

** $P < .01$.

*** $P < .001$.

attempts, those from the competition treatment had a significantly lower rate of copulation attempts than control males on both day 1 ($Z = -3.63$, $P < .001$) and day 3 ($Z = -3.90$, $P < .001$; table 1; fig. 2A).

On day 1, only 13 out of 152 males (9%) displayed courtship. There was no effect of the competitive treatment on whether a male displayed ($Z = 0.29$, $P = .770$) or on his display rate ($Z = 0.81$, $P = .418$). On day 3, however, 84 out of 152 males (55.3%) performed a courtship display. Among competition treatment males, larger individuals were less likely to display, but among control males there was no effect of body size (interaction: $Z = -2.14$, $P = .032$). For males that displayed, competition treatment males performed significantly fewer courtship displays ($Z = -3.13$, $P = .002$; table 1; fig. 2B).

Sperm Production

There was no difference in sperm count between competition treatment and control males ($t = 0.34$, $P = .734$) or a detectable effect of male size ($t = 1.33$, $P = .184$; table 2; fig. 2C). However, competition treatment males had slower-swimming sperm ($t = -2.04$, $P = .043$), as did smaller males ($t = 2.82$, $P = .006$; table 2; fig. 2D).

Reproductive Success

Controlling for male morphology and heterozygosity, competition treatment males sired significantly fewer offspring than control males (intercept: $t = -2.53$, $P = .014$). Intriguingly, the proportion of offspring sired by the competition treatment males decreased significantly with female size ($t = -2.38$, $P = .020$; fig. 3). The lower reproductive success of competition treatment males was still apparent, however, even if we excluded female size from the model (intercept: $t = -2.26$, $P = .027$). In contrast, the share of paternity for competition treatment males was unaffected by how much they differed from their rival with respect to body size ($t = -1.47$, $P = .144$), heterozygosity ($t = 1.10$, $P = .275$), or relative gonopodium length ($t = 0.19$, $P = .847$; table 3).

Discussion

On the basis of general sexual selection theory, we predicted that male *Gambusia holbrooki* previously exposed to cues from a rival would plastically invest more into sexually selected traits. This should lead to greater mating success and/or higher sperm competitiveness for these males compared with control males (Bretman et al. 2011a; Kelly and Jennions 2011). The optimal allocation to sexual traits, however, is contingent on how rivals affect both the relationship between mating effort and mating success and that between sperm traits and ejaculate competitiveness. That is, the relative marginal gains of investment into each trait depend on how it affects net reproductive success (Parker et al. 2013). When two *G. holbrooki* males competed for a female, the male that had experienced social cues indicative of more sexual competition (competition treatment) was significantly less likely to perform courtship displays and attempt to mate than a male who had not experienced these cues (control). In addition, competition treatment males had the same sperm reserves but significantly slower-swimming sperm than control males. This resulted in competition treatment males siring significantly fewer offspring. The unanticipated outcome of exposure to cues from rivals was therefore making males less, rather than more, reproductively successful.

Table 3: Effects of competition treatment, female size, heterozygosity, relative gonopodium length, and male size on paternity

	Paternity (estimate \pm SE)
Intercept	-.57 \pm .23*
Δ Heterozygosity	.22 \pm .20
Δ Relative gonopodium	.04 \pm .20
Δ Male size (SL)	-.94 \pm .63
Female size	-.52 \pm .22*

Note: Model details are given in the main text. $N = 74$ pairs. Heterozygosity, relative gonopodium length, and male body size are used in this model as the differences (competition treatment - control) between the paired males. Paternity is the proportion of offspring sired by the competition treatment male. SL = standard length.

* $P < .05$.

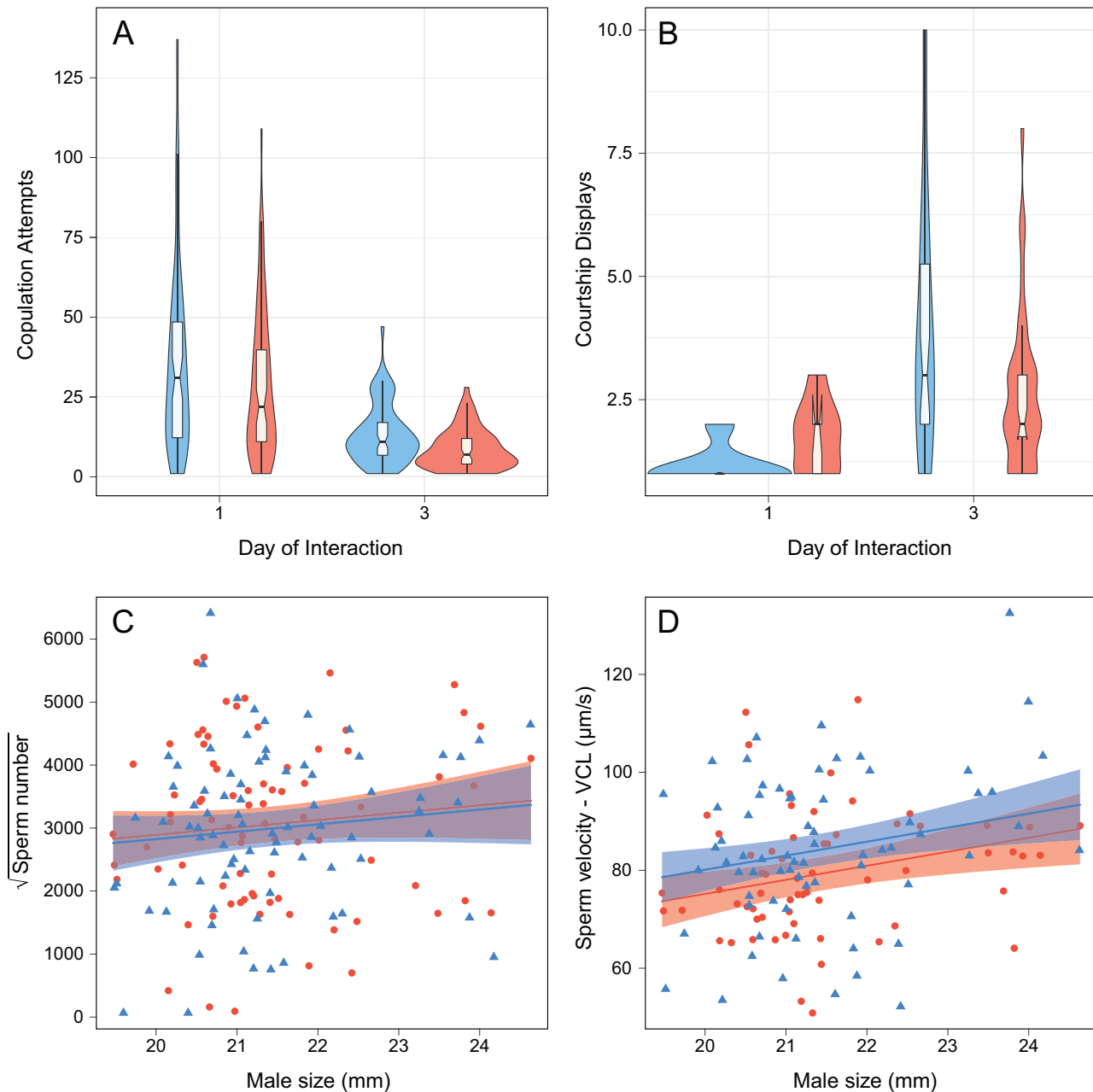


Figure 2: Effect of competition on mating behavior (A, B) and sperm production (C, D). Violin plots for the number of copulation attempts (A) and violin plots for the number of courtship displays (B) that competition treatment (in red) and control (in blue) males performed on days 1 and 3 of the behavioral trials. Boxplots indicate the median value and interquartile range. The notch represents a 95% confidence interval for the median. The horizontal width of the plot represents the kernel probability density of the data along the Y-axis. C, Relationship between male body size and sperm quantity. D, Relationship between male body size and sperm velocity (curvilinear velocity; VCL). Lines represent model estimates with 95% confidence intervals. Symbols represent partial model residuals. Red represents the competition treatment, and blue represents the control treatment.

Mating Behavior

Theory generally predicts that males will allocate more resources to sexually selected traits that increase the like-

lihood of mate acquisition when the level of mating competition increases (Kokko et al. 2012). In our study, however, competition treatment males engaged in significantly fewer courtship displays and copulation attempts (i.e.,

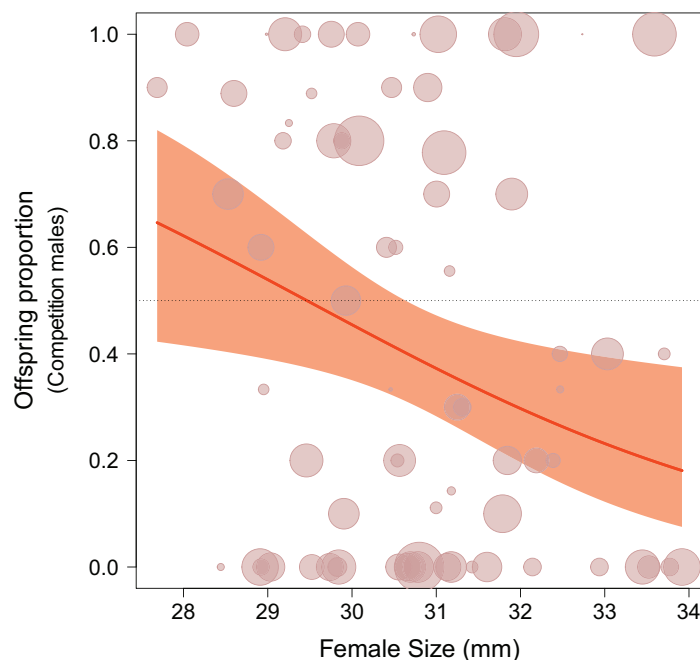


Figure 3: Relationship between female body size and the proportion of offspring sired by competition treatment males. The line represents model estimates with Wald confidence intervals. Symbols represent the raw data, and the size of each point scales to the total number of offspring produced by each female.

gonopodial thrusts) than control males. In *G. holbrooki*, the rate of attempted copulations is related to the time spent near females (Vega-Trejo et al. 2014), which at low densities, as in our behavioral trials, partly depends on the ability to chase off rivals (Fox et al. 2019). Inspection of additional responses from our collected data confirmed that, on average and relative to the control, the competition treatment males spent less time chasing their rival, spent less time near the female, and were socially subordinate (see supplemental PDF). Studies of other poeciliids have shown that when rivals are visible, males spend less time in association with a female (grijalva mosquitofish, *Heterophallus milleri*; Ziege et al. 2008) and reduce their mating effort (Atlantic molly, *Poecilia mexicana*; Plath et al. 2008). In contrast, studies on yet other poeciliids have shown that when competitors are visible or present, males court more frequently (swordtail fish, *Xiphophorus birchmanni*; Fisher and Rosenthal 2006), spend more time pursuing females (Trinidadian guppy, *Poecilia reticulata*; Magellan et al. 2005), and increase their mating effort (sailfin molly, *Poecilia latipinna*; Makowicz et al. 2010). In neither case, however, are these responses to the immediate presence of a rival the same as a difference in mating behavior attributable to different past encounter rates with rivals. Studies across taxa also tend to report a general decrease in courtship as a plastic male response to cues about higher mat-

ing competition (review: Bretman et al. 2011a). Furthermore, there is a trend for reduced courtship when the sex ratio is more male biased (i.e., higher mating competition; meta-analysis: Weir et al. 2011), but this could be because of male-male interference rather than strategic reduction of courtship.

Male fitness depends on the net effect of all traits that affect reproductive success. Consequently, the observed reduction in courtship or copulation rates in *G. holbrooki* will not necessarily lower fitness if there are compensatory gains from greater investment into other traits. For example, in many poeciliids there is a shift from courtship to “sneaking” when rivals are present (e.g., guppies, *P. reticulata*; Evans and Magurran 1999; Devigili et al. 2015; Catterlan et al. 2016). However, there were no detectable trade-offs involving greater investment into other behavioral traits in *G. holbrooki*. There was a reduction in both copulation attempts and courtship displays by the competitive treatment males and a decline in the rate at which these males chased their rival to gain proximity to the female.

Sperm Production

Theory predicts that males increase sperm production under elevated sperm competition (Parker and Pizzari 2010; Parker et al. 2013), but our control and competition treatment

males had similar sperm reserves. One explanation for this is that selection on other traits covaries with the presence of rivals and favors allocation to these traits because of higher marginal fitness gains (Lüpold et al. 2014; reviewed in Simmons et al. 2017). In *G. holbrooki*, we can rule out greater allocation to mating behavior. We cannot, however, preclude greater investment into seminal components (e.g., Hopkins et al. 2019), nor can we preclude the possibility that males adjust their ejaculate size (Wedell et al. 2002; Evans et al. 2003; Kelly and Jennions 2011).

Aside from sperm quantity, males can also adjust their expenditure on traits under sperm competition by altering sperm quality (e.g., length or velocity; Immler et al. 2010). More motile, faster-swimming sperm are often better at fertilization (Snook 2005), although there can be a trade-off between sperm velocity and longevity that affects fertilization success after prolonged sperm storage (e.g., Smith 2012). We found, however, that competition treatment males had significantly slower-swimming sperm than control males. Few comparable studies test whether exposure to social cues indicative of elevated sperm competition affect sperm velocity, and many of them do not expose males to cues from rivals for a full spermatogenesis cycle. Yet sperm velocity is largely determined by sperm morphology (Lüpold et al. 2009; Firman and Simmons 2010), which in turn is shaped during spermatogenesis (Schulz et al. 2010). We suggest that future studies should increase the duration of exposure to social cues to test for changes in sperm velocity or other sperm quality traits (Rouse and Bretman 2016).

Reproductive Success

The most important aspect of our study was to test whether exposure to social cues affected male reproductive success. The observed decline in mating performance and sperm velocity of competition treatment males suggested that they were less competitive than control males. However, trade-offs among traits (Simmons et al. 2017), combined with female control of mating and fertilization (e.g., Bretman et al. 2009b; Gasparini et al. 2009), make it difficult to predict male reproductive success. Many studies report plastic changes in male sexual traits in response to social cues (Kelly and Jennions 2011; Weir et al. 2011; review: Bretman et al. 2011a), but far fewer test further whether males exposed to a sexually competitive environment shift their investment in sexually selected traits that will elevate their reproductive success over that of males lacking such exposure. The available studies are all on insects (e.g., Sakaluk and Müller 2008; Bretman et al. 2009b, 2011b, 2012; Wigby et al. 2009; Barbosa 2012; Price et al. 2012).

We found that male *G. holbrooki* exposed to cues seemingly indicative of increased sexual competition had a sig-

nificantly lower share of paternity than control males. This finding is consistent with the observed effect of the social treatment on male mating behavior and sperm velocity. It also raises an obvious question: Why did males exposed to cues about the presence of rivals not reallocate their resources to sexual traits and perform better when competing for a female and fertilizations?

First, we can ask how males perceived the social cues provided. We used a common experimental design (i.e., presence or absence of visual and chemical cues from rivals) used in many sperm competition studies (e.g., Firman et al. 2018; Noguera 2019), including studies on poeciliid fish (e.g., Evans et al. 2003; Evans 2009; for a variant, see Aspbury 2007). Such studies tend to report a shift in sexual traits in the direction predicted if the presence of rivals indicate an increase in the future level of reproductive competition (e.g., greater total sperm production; Parker and Pizzari 2010; Kelly and Jennions 2011). It is possible, however, that male *G. holbrooki* did not treat the presence of a rival as indicative of greater future mating and/or sperm competition. Mosquitofish often live at high densities, and there might be a lack of selection for adaptive plasticity in response to the presence of rivals. This explanation cannot, however, account for the significantly lower reproductive success of the competition treatment males.

Second, optimal allocation of resources occurs both among traits and across life-history stages (e.g., early and late life; Lemaître et al. 2015) to determine lifetime reproductive success. Few studies, however, have investigated how exposure to cues of sexual competition affects male lifetime reproductive success (but see Bretman et al. 2013). Investigating such effects would provide a better understanding of the strategic reproductive investment of males in response to the level of sexual competition. In our study, we quantified a male's short-term reproductive success when competing for a single female. It is possible that increased reproductive competition favors lower investment per mating (Parker and Pizzari 2010), which can increase male life span (e.g., Moatt et al. 2013) and therefore elevate lifetime reproductive success.

Third, our experiment might have done more than simply change a male's perception of the future level of sexual competition. For example, prolonged exposure to rivals that have greater access to a female (i.e., competition treatment males could not interact with the female but rival males could; fig. 1) might induce a "winner-loser" type effect (Hsu et al. 2006), reducing the future success of the competition treatment males when competing for mates (see Filice and Dukas 2019). Indeed, in *G. holbrooki* losers of staged fights subsequently spend less time with a female than a size-matched winner (Harrison et al. 2018). However, using an experimental setup, where

rivals would be unable to interact with a female, might create different problems. For example, males that fail to see a rival with apparent access to a female mate might erroneously perceive him as sexually inactive (e.g., “audience” effect studies in other poeciliids; Bierbach et al. 2011; Plath and Bierbach 2011).

Finally, competition treatment males might have invested energy into attacking the rival male through the mesh partition, even though no direct physical contact was possible. This could deplete their energy reserves and affect their hormonal balance, reducing their ability to gain access to a female and sire offspring when competing with a control male that had not engaged in aggressive interactions. In some species of *Drosophila*, for example, exposure to rivals leads to a decline in the ability to acquire mates (Lizé et al. 2014; see also Tuni et al. 2017). It is noteworthy that most previous poeciliid studies testing the effect of social cues from rivals on males have used a far shorter period of exposure (i.e., days, not weeks), so any energetic or winner-loser effects are likely to be smaller in magnitude.

Future Implications: Experimental Design

It is a challenge to predict how exposure to cues from rivals affect focal traits. We rarely know the relative marginal gains from investment into different traits when both mating and sperm competition levels change. The role of mating and sperm competition in allocation plasticity is most likely to be estimated in hindsight, using the outcome of studies across many taxa that report relative changes in traits under pre- and postcopulatory sexual selection. This raises the question of how we design experiments. Here we have underlined the importance of the duration of the exposure to cues from rivals. Shorter exposure might reduce any confounding energetic or winner-loser effects, but it might also preclude effects on traits that require longer-term physiological processes (e.g., spermatogenesis). There is no easy way to determine the most biologically relevant duration of exposure to rivals. Future studies should explore potential variation in exposure length and—crucially—test whether the observed changes elevate reproductive success. This is essential to assess whether and how males adaptively adjust their sexual competitiveness in response to the future strength of sexual selection.

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Statement of Authorship

F.S. carried out the experiment. R.V.-T. collected the sperm data. M.L.H. and M.D.J. conceived the original idea. All authors contributed to the experimental design. F.S. performed the data analysis, data visualization and interpretation. R.V.-T., M.L.H., and M.D.J. contributed to data analysis and interpretation. F.S. and M.D.J. wrote the manuscript. F.S. and M.D.J. provided funding for the project. M.L.H. and M.D.J. supervised the project. All authors contributed to the revisions of the final article.

Literature Cited

- Abramoff, M. D., P. J. Magalhães, and S. J. Ram. 2004. Image processing with ImageJ. *Biophotonics International* 11:36–42.
- Aspbury, A. S. 2007. Sperm competition effects on sperm production and expenditure in sailfin mollies, *Poecilia latipinna*. *Behavioral Ecology* 18:776–780.
- Barbosa, F. 2012. Males responding to sperm competition cues have higher fertilization success in a soldier fly. *Behavioral Ecology* 23:815–819.
- Bierbach, D., A. Girmdt, S. Hamfler, M. Klein, F. Mücksch, M. Pershorn, M. Schwinn, et al. 2011. Male fish use prior knowledge about rivals to adjust their mate choice. *Biology Letters* 7:349–351.
- Billard, R., and M. P. Cosson. 1992. Some problems related to the assessment of sperm motility in freshwater fish. *Journal of Experimental Zoology* 261:122–131.
- Bisazza, A. 1993. Male competition, female mate choice and sexual size dimorphism in poeciliid fishes. *Marine Behaviour and Physiology* 23:257–286.
- Bisazza, A., and G. Marin. 1995. Sexual selection and sexual size dimorphism in the eastern mosquitofish *Gambusia holbrooki* (Pisces Poeciliidae). *Ethology Ecology and Evolution* 7:169–183.
- Blumstein, J. W., C. S. Evans, and J. C. Daniel. 2000. JWatcher 0.9: an introductory user’s guide. <http://www.jwatcher.ucla.edu>.
- Booksmythe, I., M. L. Head, J. S. Keogh, and M. D. Jennions. 2016. Fitness consequences of artificial selection on relative male genital size. *Nature Communications* 7:ncomms11597.
- Bretman, A., C. Fricke, and T. Chapman. 2009a. Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. *Proceedings of the Royal Society B* 276:1705–1711.
- Bretman, A., M. J. G. Gage, and T. Chapman. 2011a. Quick-change artists: male plastic behavioural responses to rivals. *Trends in Ecology and Evolution* 26:467–473.
- Bretman, A., D. Newcombe, and T. Tregenza. 2009b. Promiscuous females avoid inbreeding by controlling sperm storage. *Molecular Ecology* 18:3340–3345.

- Bretman, A., J. D. Westmancoat, and T. Chapman. 2013. Male control of mating duration following exposure to rivals in fruit-flies. *Journal of Insect Physiology* 59:824–827.
- Bretman, A., J. D. Westmancoat, M. J. G. Gage, and T. Chapman. 2011b. Males use multiple, redundant cues to detect mating rivals. *Current Biology* 21:617–622.
- . 2012. Individual plastic responses by males to rivals reveal mismatches between behaviour and fitness outcomes. *Proceedings of the Royal Society B* 279:2868–2876.
- Cattelan, S., J. P. Evans, A. Pilastro, and C. Gasparini. 2016. The effect of sperm production and mate availability on patterns of alternative mating tactics in the guppy. *Animal Behaviour* 112:105–110.
- Cohen, J. 1988. *Statistical power analysis for the behavioral sciences*. 2nd ed. Lawrence Erlbaum, Hillsdale, NJ.
- Constantz, G. D. 1989. Reproductive biology of poeciliid fishes. Pages 33–50 in G. K. Meffe and F. F. Snelson, Jr., eds. *Ecology and evolution of livebearing fishes (Poeciliidae)*. Prentice Hall, Englewood Cliffs, NJ.
- Devigili, A., V. Doldán-Martelli, and A. Pilastro. 2015. Exploring simultaneous allocation to mating effort, sperm production, and body growth in male guppies. *Behavioral Ecology* 26:1203–1211.
- Dore, A. A., L. McDowall, J. Rouse, A. Bretman, M. J. G. Gage, and T. Chapman. 2018. The role of complex cues in social and reproductive plasticity. *Behavioral Ecology and Sociobiology* 72:124.
- Dziewieczynski, T. L., S. Lyman, and E. A. Poor. 2009. Male Siamese fighting fish, *Betta splendens*, increase rather than conceal courtship behavior when a rival is present. *Ethology* 115:186–195.
- Edward, D. A., and T. Chapman. 2011. The evolution and significance of male mate choice. *Trends in Ecology and Evolution* 26:647–654.
- Emlen, D. J. 2008. The evolution of animal weapons. *Annual Review of Ecology, Evolution, and Systematics* 39:387–413.
- Evans, J. P. 2009. No evidence for sperm priming responses under varying sperm competition risk or intensity in guppies. *Naturwissenschaften* 96:771–779.
- Evans, J. P., and F. Garcia-Gonzalez. 2016. The total opportunity for sexual selection and the integration of pre- and post-mating episodes of sexual selection in a complex world. *Journal of Evolutionary Biology* 29:2338–2361.
- Evans, J. P., and A. E. Magurran. 1999. Male mating behaviour and sperm production characteristics under varying sperm competition risk in guppies. *Animal Behaviour* 58:1001–1006.
- Evans, J. P., M. Pierotti, and A. Pilastro. 2003. Male mating behaviour and ejaculate expenditure under sperm competition risk in the eastern mosquitofish. *Behavioral Ecology* 14:268–273.
- Filice, D. C. S., and R. Dukas. 2019. Winners have higher precopulatory mating success but losers have better post-copulatory outcomes. *Proceedings of the Royal Society B* 286:20182838.
- Firman, R. C., F. Garcia-Gonzalez, L. W. Simmons, and G. I. André. 2018. A competitive environment influences sperm production, but not testes tissue composition, in house mice. *Journal of Evolutionary Biology* 31:1647–1654.
- Firman, R. C., and L. W. Simmons. 2010. Sperm midpiece length predicts sperm swimming velocity in house mice. *Biology Letters* 6:513–516.
- Fisher, H. S., and G. G. Rosenthal. 2006. Male swordtails court with an audience in mind. *Biology Letters* 3:5–7.
- Fox, R. J., E. E. Gearing, M. D. Jennions, and M. L. Head. 2019. Variation in the condition-dependence of individual sexual traits in male eastern mosquitofish, *Gambusia holbrooki*. *Behavioral Ecology* 30:666–674.
- Fromhage, L., J. M. McNamara, A. I. Houston, and A. E. and E. M. C. Whitlock. 2008. Sperm allocation strategies and female resistance: a unifying perspective. *American Naturalist* 172:25–33.
- Gardiner, D. M. 1978. Utilization of extracellular glucose by spermatozoa of two viviparous fishes. *Comparative Biochemistry and Physiology A* 59:165–168.
- Gasparini, C., A. V. Peretti, and A. Pilastro. 2009. Female presence influences sperm velocity in the guppy. *Biology Letters* 5:792–794.
- Hardy, I. C. W., and M. Briffa, eds. 2013. *Animal contests*. Cambridge University Press, Cambridge.
- Harrison, L. M., M. D. Jennions, and M. L. Head. 2018. Does the winner-loser effect determine male mating success? *Biology Letters* 14:20180195.
- Harrison, X. A. 2014. Using observation-level random effects to model overdispersion in count data in ecology and evolution. *PeerJ* 2:e616.
- Hartig, F. 2016. DHARMA: residual diagnostics for hierarchical (multi-level/mixed) regression models. R package version 0.1.0. <http://CRAN.R-project.org/package=DHARMA>.
- Head, M. L., A. T. Kahn, J. M. Henshaw, J. S. Keogh, and M. D. Jennions. 2017. Sexual selection on male body size, genital length and heterozygosity: consistency across habitats and social settings. *Journal of Animal Ecology* 86:1458–1468.
- Hoi, H., H. Tost, and M. Griggio. 2011. The effect of breeding density and male quality on paternity-assurance behaviours in the house sparrow, *Passer domesticus*. *Journal of Ethology* 29:31–38.
- Hooper, A. K., J. Lehtonen, L. E. Schwanz, and R. Bonduriansky. 2018. Sexual competition and the evolution of condition-dependent ageing. *Evolution Letters* 2:37–48.
- Hopkins, B. R., I. Sepil, M.-L. Thézénas, J. F. Craig, T. Miller, P. D. Charles, R. Fischer, et al. 2019. Divergent allocation of sperm and the seminal proteome along a competition gradient in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the USA* 116:17925–17933.
- Hsu, Y., R. L. Earley, and L. L. Wolf. 2006. Modulation of aggressive behaviour by fighting experience: mechanisms and contest outcomes. *Biological Reviews* 81:33–74.
- Iglesias-Carrasco, M., R. J. Fox, A. Vincent, M. L. Head, and M. D. Jennions. 2019. No evidence that male sexual experience increases mating success in a coercive mating system. *Animal Behaviour* 150:201–208.
- Immler, S., S. R. Pryke, T. R. Birkhead, and S. C. Griffith. 2010. Pronounced within-individual plasticity in sperm morphometry across social environments. *Evolution* 64:1634–1643.
- Kahn, A. T., H. Kokko, and M. D. Jennions. 2013. Adaptive sex allocation in anticipation of changes in offspring mating opportunities. *Nature Communications* 4:1603.
- Kelly, C. D., and M. D. Jennions. 2011. Sexual selection and sperm quantity: meta-analyses of strategic ejaculation. *Biological Reviews* 86:863–884.
- Kokko, H., M. D. Jennions, and R. Brooks. 2006. Unifying and testing models of sexual selection. *Annual Review of Ecology, Evolution, and Systematics* 37:43–66.
- Kokko, H., H. Klug, and M. D. Jennions. 2012. Unifying cornerstones of sexual selection: operational sex ratio, Bateman gradient and the scope for competitive investment. *Ecology Letters* 15:1340–1351.
- Koya, Y., and A. Iwase. 2004. Annual reproductive cycle and rate of the spermatogenic process in male mosquitofish *Gambusia affinis*. *Ichthyological Research* 51:131–136.

- Kuijper, B., I. Pen, and F. J. Weissing. 2012. A guide to sexual selection theory. *Annual Review of Ecology, Evolution, and Systematics* 43:287–311.
- Lemaître, J.-F., V. Berger, C. Bonenfant, M. Douhard, M. Gamelon, F. Plard, and J.-M. Gaillard. 2015. Early-late life trade-offs and the evolution of ageing in the wild. *Proceedings of the Royal Society B* 282:20150209.
- Lizé, A., T. A. R. Price, C. Heys, Z. Lewis, and G. D. D. Hurst. 2014. Extreme cost of rivalry in a monandrous species: male-male interactions result in failure to acquire mates and reduced longevity. *Proceedings of the Royal Society B* 281:20140631.
- Lüpold, S., S. Calhim, S. Immler, and T. R. Birkhead. 2009. Sperm morphology and sperm velocity in passerine birds. *Proceedings of the Royal Society B* 276:1175–1181.
- Lüpold, S., M. K. Manier, N. Puniamoorthy, C. Schoff, W. T. Starmer, S. H. B. Luepold, J. M. Belote, et al. 2016. How sexual selection can drive the evolution of costly sperm ornamentation. *Nature* 533:535–538.
- Lüpold, S., J. L. Tomkins, L. W. Simmons, and J. L. Fitzpatrick. 2014. Female monopolization mediates the relationship between pre- and postcopulatory sexual traits. *Nature Communications* 5:3184.
- Magellan, K., L. B. Pettersson, and A. E. Magurran. 2005. Quantifying male attractiveness and mating behaviour through phenotypic size manipulation in the Trinidadian guppy, *Poecilia reticulata*. *Behavioral Ecology and Sociobiology* 58:366–374.
- Makowicz, A. M., M. Plath, and I. Schlupp. 2010. Using video playback to study the effect of an audience on male mating behavior in the sailfin molly (*Poecilia latipinna*). *Behavioural Processes* 85:36–41.
- Marsh, J. N., R. Vega-Trejo, M. D. Jennions, and M. L. Head. 2017. Why does inbreeding reduce male paternity? effects on sexually selected traits. *Evolution* 71:2728–2737.
- Moatt, J. P., C. Dytham, and M. D. F. Thom. 2013. Exposure to sperm competition risk improves survival of virgin males. *Biology Letters* 9:20121188.
- Nakagawa, S., and H. Schielzeth. 2010. Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. *Biological Reviews* 85:935–956.
- Noguera, J. C. 2019. Crickets increase sexual signalling and sperm protection but live shorter in the presence of rivals. *Journal of Evolutionary Biology* 32:49–57.
- O’Dea, R. E., M. D. Jennions, and M. L. Head. 2014. Male body size and condition affects sperm number and production rates in mosquitofish, *Gambusia holbrooki*. *Journal of Evolutionary Biology* 27:2739–2744.
- Órfão, I., A. F. Ojanguren, M. Barbosa, L. Vicente, S. A. M. Varela, and A. E. Magurran. 2018. How pre- and postcopulatory sexual selection influence male mating decisions in a promiscuous species. *Animal Behaviour* 136:147–157.
- Parker, G. A., C. M. Lessells, and L. W. Simmons. 2013. Sperm competition games: a general model for precopulatory male-male competition. *Evolution* 67:95–109.
- Parker, G. A., and T. Pizzari. 2010. Sperm competition and ejaculate economics. *Biological Reviews* 85:897–934.
- Peden, A. E. 1970. Courtship behaviour of *Gambusia* (Poeciliidae) with emphasis on isolating mechanisms. PhD diss. University of Texas, Austin.
- Pilastro, A., E. Giacomello, and A. Bisazza. 1997. Sexual selection for small size in male mosquitofish (*Gambusia holbrooki*). *Proceedings of the Royal Society B* 264:1125–1129.
- Plath, M., and D. Bierbach. 2011. Sex and the public. *Communicative and Integrative Biology* 4:276–280.
- Plath, M., S. Richter, R. Tiedemann, and I. Schlupp. 2008. Male fish deceive competitors about mating preferences. *Current Biology* 18:1138–1141.
- Price, T. A. R., A. Lizé, M. Marcello, and A. Bretman. 2012. Experience of mating rivals causes males to modulate sperm transfer in the fly *Drosophila pseudoobscura*. *Journal of Insect Physiology* 58:1669–1675.
- Punzalan, D., F. H. Rodd, and L. Rowe. 2010. Temporally variable multivariate sexual selection on sexually dimorphic traits in a wild insect population. *American Naturalist* 175:401–414.
- Pyke, G. H. 2005. A review of the biology of *Gambusia affinis* and *G. holbrooki*. *Reviews in Fish Biology and Fisheries* 15:339–365.
- Ramm, S. A., and P. Stockley. 2009. Adaptive plasticity of mammalian sperm production in response to social experience. *Proceedings of the Royal Society B* 276:745–751.
- R Development Core Team. 2017. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. Version 3.3.1. <http://www.R-project.org>.
- Rouse, J., and A. Bretman. 2016. Exposure time to rivals and sensory cues affect how quickly males respond to changes in sperm competition threat. *Animal Behaviour* 122:1–8.
- Rouse, J., K. Watkinson, and A. Bretman. 2018. Flexible memory controls sperm competition responses in male *Drosophila melanogaster*. *Proceedings of the Royal Society B* 285:20180619.
- Sakaluk, S. K., and J. K. Müller. 2008. Risk of sperm competition mediates copulation duration, but not paternity, of male burying beetles. *Journal of Insect Behavior* 21:153–163.
- Schielzeth, H. 2010. Simple means to improve the interpretability of regression coefficients: interpretation of regression coefficients. *Methods in Ecology and Evolution* 1:103–113.
- Schulz, R. W., L. R. de França, J.-J. Lareyre, F. LeGac, H. Chiarini-Garcia, R. H. Nobrega, and T. Miura. 2010. Spermatogenesis in fish. *General and Comparative Endocrinology* 165:390–411.
- Simmons, L. W., S. Lüpold, and J. L. Fitzpatrick. 2017. Evolutionary trade-off between secondary sexual traits and ejaculates. *Trends in Ecology and Evolution* 32:964–976.
- Sloan, N. S., M. Lovegrove, and L. W. Simmons. 2018. Social manipulation of sperm competition intensity reduces seminal fluid gene expression. *Biology Letters* 14:20170659.
- Smith, C. C. 2012. Opposing effects of sperm viability and velocity on the outcome of sperm competition. *Behavioral Ecology* 23:820–826.
- Snook, R. R. 2005. Sperm in competition: not playing by the numbers. *Trends in Ecology and Evolution* 20:46–53.
- Spagopoulou, F., R. Vega-Trejo, M. L. Head, M. D. Jennions. 2020. Data from: Shifts in reproductive investment in response to competitors lowers male reproductive success. *American Naturalist*, Dryad Digital Repository, <https://doi.org/10.5061/dryad.v41ns1rs9>.
- Tazzyman, S. J., T. Pizzari, R. M. Seymour, and A. Pomiankowski. 2009. The evolution of continuous variation in ejaculate expenditure strategy. *American Naturalist* 174:E71–E82.
- Tuni, C., S. Weber, T. Bilde, and G. Uhl. 2017. Male spiders reduce pre- and postmating sexual investment in response to sperm competition risk. *Behavioral Ecology* 28:1030–1036.
- Vega-Trejo, R., M. L. Head, J. S. Keogh, and M. D. Jennions. 2017. Experimental evidence for sexual selection against inbred males. *Journal of Animal Ecology* 86:394–404.
- Vega-Trejo, R., R. E. O’Dea, M. D. Jennions, and M. L. Head. 2014. The effects of familiarity and mating experience on mate

- choice in mosquitofish, *Gambusia holbrooki*. *Behavioral Ecology* 25:1205–1211.
- Wang, S., M. Cummings, and M. Kirkpatrick. 2015. Coevolution of male courtship and sexual conflict characters in mosquitofish. *Behavioral Ecology* 26:1013–1020.
- Wedell, N., M. J. G. Gage, and G. A. Parker. 2002. Sperm competition, male prudence and sperm-limited females. *Trends in Ecology and Evolution* 17:313–320.
- Weir, L. K., J. W. A. Grant, and J. A. Hutchings. 2011. The influence of operational sex ratio on the intensity of competition for mates. *American Naturalist* 177:167–176.
- Wigby, S., L. K. Sirot, J. R. Linklater, N. Buehner, F. C. F. Calboli, A. Bretman, M. F. Wolfner, et al. 2009. Seminal fluid protein allocation and male reproductive success. *Current Biology* 19: 751–757.
- Williams, P. D., T. Day, and E. Cameron. 2005. The evolution of sperm-allocation strategies and the degree of sperm competition. *Evolution* 59:492–499.
- Wilson, R. S. 2005. Temperature influences the coercive mating and swimming performance of male eastern mosquitofish. *Animal Behaviour* 70:1387–1394.
- Zane, L., W. S. Nelson, A. G. Jones, and J. C. Avise. 1999. Microsatellite assessment of multiple paternity in natural populations of a live-bearing fish, *Gambusia holbrooki*. *Journal of Evolutionary Biology* 12:61–69.
- Ziege, M., L. Padur, V. Duwe, A. Ramm, R. Riesch, and M. Plath. 2008. Audience effect alters mate choice in male *Heterophallus milleri* (Poeciliidae). *Bulletin in Fish Biology* 10:7.
- genome generated by reduced representation shotgun sequencing. *Nature* 407:513–516.
- Baird, N. A., P. D. Etter, T. S. Atwood, M. C. Currey, A. L. Shiver, Z. A. Lewis, E. U. Selker, W. A. Cresko, and E. A. Johnson. 2008. Rapid SNP discovery and genetic mapping using sequenced RAD markers. *PLoS ONE* 3:e3376.
- Courtois, B., A. Audebert, A. Dardou, S. Roques, T.-G. Herrera, G. Droc, J. Frouin, et al. 2013. Genome-wide association mapping of root traits in a japonica rice panel. *PLoS ONE* 8:e78037.
- Cruz, V. M. V., A. Kilian, and D. A. Dierig. 2013. Development of DArT marker platforms and genetic diversity assessment of the U.S. collection of the new oilseed crop *Lesquerella* and related species. *PLoS ONE* 8:e64062.
- Elshire, R. J., J. C. Glaubitz, Q. Sun, J. A. Poland, K. Kawamoto, E. S. Buckler, and S. E. Mitchell. 2011. A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLOS ONE* 6:e19379.
- Hu, H., X. Liu, W. Jin, H. Hilger Ropers, and T. F. Wienker. 2015. Evaluating information content of SNPs for sample-tagging in re-sequencing projects. *Scientific Reports* 5:10247.
- Kilian, A., P. Wenzl, E. Huttner, J. Carling, L. Xia, H. Blois, V. Caig, et al. 2012. Diversity arrays technology: a generic genome profiling technology on open platforms. Pages 67–89 in F. Pompanon and A. Bonin, eds. *Data production and analysis in population genomics: methods and protocols*. Humana, Totowa, NJ.
- Raman, H., R. Raman, A. Kilian, F. Detering, J. Carling, N. Coombes, S. Diffey, et al. 2014. Genome-wide delineation of natural variation for pod shatter resistance in *Brassica napus*. *PLoS ONE* 9:e101673.

References Cited Only in the Online Enhancements

- Altshuler, D., V. J. Pollara, C. R. Cowles, W. J. V. Etten, J. Baldwin, L. Linton, and E. S. Lander. 2000. An SNP map of the human

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Gambusia holbrooki mating attempt, with male (bottom) performing a gonopodial thrust, by swinging his gonopodium (an intromittent organ modified from the anal fin) forward and trying to insert it into the gonopore of the female (top). Photo credit: Ellen Gearing.