



Novel ablation technique shows no sperm priming response by male eastern mosquitofish to cues of female availability

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Abstract

Changes in mate availability and sperm competition should generate selection to adjust investments into different pre- and post-copulatory traits so that the product of mating and fertilization success maximize net male reproductive success. Given costly sperm production and the risk of sperm depletion, males should invest strategically in ejaculates. Here, we use the eastern mosquitofish (*Gambusia holbrooki*), where males have a single coercive mating tactic, to test whether the number of cues indicative of female availability affects the rate of sperm available for mating (so-called sperm priming). We also tested whether plasticity in sperm production varies with male body size. We created four socio-sexual treatments that differ in the number of female-derived cues: none, chemical, chemical and visual, and full access to a female. We used ablation surgery, removing the tip of the male gonopodium (intromittent organ), to prevent males from mating with a female in the treatment where they interacted with females. We hypothesized that elevated sperm priming would be associated with more cues about female availability, and be more apparent in smaller, subordinate males due to their lower baseline sperm count (higher risk of sperm depletion) and their potential disadvantage during premating competition (leading to higher marginal benefits from sperm investment). There was, however, no evidence for sperm priming. The rate of sperm availability for mating and the baseline sperm reserves were, however, dependent on male body size. We discuss possible reasons for our findings. We also note that our study provides novel insights into the proximate mechanisms associated with sperm release in Poeciliids. Our confirmation of the fact that removing the gonopodium tip prevents a male from releasing sperm when housed with a female has many potential applications (e.g., in the study of effects of ejaculate investment and mating effort on male mating success and longevity).

Significance statement

When ejaculates are energetically costly, males should strategically adjust sperm production in response to relevant social cues such as female availability. Using four socio-sexual scenarios, we demonstrate that male mosquitofish *G. holbrooki* do not produce less sperm when housed alone, compared to being exposed only to chemical cues from females, or to chemical and visual cues, or even when allowed full access to a female. The absence of plasticity in sperm production, when compared to that reported in other Poeciliid fishes, suggests that the relationship between sperm number and mating success might depend upon the mating system. If increased sperm number has a small effect on male reproductive success, there might not be selection for plastic shifts in sperm production. We also showed that ablation surgery on the male's gonopodium allows us to let males interact with females without ejaculating.

Keywords Sperm priming · Poeciliidae · Sexual selection · Ejaculate economics · Gonopodium · Sperm competition

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Introduction

Males sometimes strategically adjust their investment into sperm production in response to the social contexts (review: Evans and Garcia-Gonzalez 2016), but the extent to which this adaptive plasticity varies predictably among males (e.g., shows condition dependence) is understudied (but see Cornwallis and Birkhead 2007; Cattelan and Pilastro 2018).

This oversight is surprising given that sperm is energetically costly to produce, and can take a substantial time to replenish (Thomsen et al. 2006; Uma and Sevgili 2015). For example, male adders (*Vipera berus*) lose significant body mass during spermatogenesis (Olsson et al. 1997), equivalent to the energy invested in all other mating activities combined (e.g., mate-searching, competition, courtships). Similarly, spermatogenesis by male nematodes (*Caenorhabditis elegans*) reduces their lifespan by 65% compared to males who do not develop viable sperm (Van Voorhies 1992). In addition, the time taken to replenish sperm reserves removes males from the mating pool, and can lead to lost mating opportunities (e.g., three to nine days for rats, Jackson and Dewsbury 1979), and/or a decline in the sperm number per ejaculate, lowering male sperm competitiveness (e.g., Preston et al. 2001).

Strategic sperm production, male mate choice, and adjustment of ejaculate size in response to the risk of future sperm limitation can be important factors affecting male fitness (review: Wedell et al. 2002). This is particularly true in species where females mate multiply. Males are expected to evolve the ability to adjust sperm production and allocation in response to the perceived likelihood of future mating opportunity (e.g., based on female cues; Reinhold et al. 2002); the level of sperm competition (e.g., the presence and/or density of rivals; Parker 1990); and their competitiveness (e.g., relative body size or physical condition; Pitnick and Markow 1994). For example, male cichlids (*Lamprologus callipterus*) reduce spawning duration and the number of ejaculations per female when female availability is high (Schütz et al. 2017). In livebearer fishes (family: Poeciliidae), there is evidence from several species that sperm reserves vary with a male's energetic resources (e.g., depend on his body size, diet, and food availability; Rahman et al. 2014a, b; meta-analysis: Macartney et al. 2019). There is also evidence that adaptive sperm allocation occurs in response to social cues from females (Bozynski and Liley 2003; Aspbury and Gabor 2004). The interaction between these two sources of variation in sperm production is, however, poorly studied.

It is typically assumed that adaptive sperm allocation reflects a trade-off between investment in pre-copulatory and post-copulatory sexually selected traits (Lüpold et al. 2014; review: Simmons et al. 2017). This could further promote shifts in investment into different types of traits that are under the same type of sexual selection (e.g., pre-copulatory). For example, in guppies (*Poecilia reticulata*), males in environments that increase investment into sperm production also shift away from energetically costly courtship displays towards coercive mating attempts (Cattelan et al. 2016). The optimal allocation of resources to sperm production depends on the marginal rates of return from different pre-copulatory and post-copulatory traits (Parker et al. 2013). These rates are, however, likely to depend on a male's own status and his immediate social conditions (e.g., level of mating

competition, female availability for mating; Gasparini et al. 2009; Lüpold et al. 2011, 2012; Fox et al. 2019).

Several studies have shown that social cues affect the rate of sperm available for copulation leading to so-called sperm priming. In poeciliids, for example, exposure to females for several days results in faster sperm maturation in both guppies and sailfin mollies (*Poecilia latipinna*) (Bozynski and Liley 2003; Aspbury and Gabor 2004). This sperm priming has been interpreted as an adaptive response to the greater likelihood of opportunities to inseminate females, hence the need to ejaculate more often, thereby avoiding sperm depletion (Preston et al. 2001; Cattelan and Pilastro 2018). There is, however, limited evidence in poeciliids as to whether (1) sperm priming occurs in species with a relatively low-cost mating strategy (i.e., species lacking male courtship); (2) males with less energy reserves and a lower maximum sperm number (e.g., smaller males), in which the cost of sperm production and the risk of sperm depletion is much higher, show greater plasticity in sperm priming (Aspbury and Gabor 2004; Smith and Ryan 2011; Cattelan and Pilastro 2018); and (3) the number or type of mating cues available affect a male's rate of making sperm available for mating. Previous studies have only examined the effect on sperm priming of the combination of visual and chemical cues from females (Bozynski and Liley 2003; Aspbury and Gabor 2004; Gasparini et al. 2009; Cattelan et al. 2016; but see Evans 2009). However, male assessment of the future potential for mating opportunities in Poeciliid fishes might be based on chemical cues only (Wong et al. 2005) and/or involve direct interactions with females (Killen et al. 2016).

To test for sperm priming in a species with a single male mating tactic, we studied an internally fertilized fish, the eastern mosquitofish (*Gambusia holbrooki*). Unlike guppies, male mosquitofish almost never court females, and mating is coercive (Pilastro et al. 1997). The male approaches the female from behind, swings his gonopodium (a modified anal fin) forward and then darts forward at speed to insert his gonopodium into her gonopore (Langerhans 2011). The frequency of male mating attempts is extremely high: up to one attempt per minute (e.g., Wilson 2005), and complete replenishment of sperm reserves takes several days, which is far longer than the average interval between encounters with prospective mates (O'Dea et al. 2014). This suggests that males are likely to be sperm limited unless they adjust ejaculate size and/or sperm priming in response to female availability. Male *G. holbrooki* can recognize the presence of females based on both chemical (Park and Propper 2002) and visual cues (Kodama et al. 2008). Males can then gain direct information about female availability based on the rate at which they can make copulatory attempts. These three types of cues—olfactory, visual, and behavioral—provide different types of information that might vary in its reliability and affect sperm priming. We assume the possibility of successful copulation increases with higher levels of female cues (e.g., when males start to chase

or follow a female the likelihood of ejaculation is higher than when detecting an olfactory cue). We predicted that males would regulate the allocation of sperm number in response to variation in the number of cues from females that are indicative of relative mating opportunities to reduce the risk of sperm depletion. Given that body size affects a male's success in approaching females (Pilastro et al. 1997) and winning fights (Harrison et al. 2018), we also predicted that body size would have a moderating effect on sperm priming. That is, smaller, subordinate males who (1) face a higher risk of sperm competition due to their lower social status (Bisazza et al. 2001; Smith and Ryan 2011), (2) have fewer resources to invest in ejaculates (Pitnick and Markow 1994), and (3) have lower baseline sperm production (O'Dea et al. 2014; Cattelan and Pilastro 2018) were assumed to have stronger sperm priming in response to female cues about mating opportunities.

We examined the effects of mating cues on sperm priming responses in *G. holbrooki* by placing males into four different treatments for four days: (1) no female cues, (2) only chemical cues from a female, (3) chemical and visual cues from a female, (4) visual, chemical, and behavioral cues from a female. We then measured sperm availability. Although other studies of Poeciliid sperm priming have used a 7-day exposure period (e.g., Aspbury and Gabor 2004; Cattelan et al. 2016), to ensure that sperm replenishment was still ongoing at the time of measurement, we considered four days of stimulus exposure as appropriate to reveal any difference in the rate of sperm priming because sperm reserves will not yet have reached their maximum in *G. holbrooki* (O'Dea et al. 2014). To create the fourth treatment where a male could interact with a female but we could still measure his sperm production (i.e., he could interact with a female but would not be able to release sperm), males had the tip of their gonopodium surgically removed. The distal part of the gonopodium tip has several holdfasts (e.g., claws, hooks) that may facilitate a successful copulation and sperm release, but empirical evidence is needed (Rosen and Gordon 1953; Peden 1972). We assumed and then tested that (a) surgery would not affect the rate of sperm production, but would simply (b) deprive males of tactile stimuli associated with inserting the gonopodium tip into the female gonopore that lead to ejaculation. We confirmed the effectiveness of ablation surgery in blocking sperm release by male mosquitofish. This method of gonopodium ablation is therefore a valuable tool for any future studies seeking to manipulate male expenditure on sperm production/replenishment.

Materials and methods

Origin and maintenance of experimental fish

Adult eastern mosquitofish (*Gambusia holbrooki*) were collected in Canberra, Australia (35° 18' 27" S 149° 07' 27.9" E) during March–April 2019 and taken to aquarium facilities

at the Australian National University. Stimulus females were housed in single-sex 90-L aquaria (40–50 females/tank) for at least 4 weeks, to ensure that they were not gravid prior to the start of the experiment. We maintained the fish under 14:10 L:D photoperiod at 28 °C (± 1 °C), and fed them twice daily ad libitum with brine shrimp (*Artemia salina* nauplii) and commercial fish flakes (prior to experiment), or brine shrimp alone (during experiment).

Experiment 1: effect of gonopodium tip ablation surgery on sperm priming and release

To proceed with the main part of our study, in which the sperm priming response of an ablated male experiencing visual, chemical, and behavioral cues from a female is compared to that of intact males experiencing chemical, visual and chemical, or no cues at all from a female, we needed to verify two assumptions. First, that there is no effect of ablation surgery on rates of male sperm priming. Second, that ablated males that interact with a female do not release sperm.

To test the first assumption, we ran a treatment of exposure to chemical and visual female cues (treatment 3 see below) on a group of ablated and intact (control) males. Each male was anesthetized in ice water (10s), placed on a glass slide alongside a 10-mm scale bar and photographed laterally. Photographs were subsequently analyzed using the software *ImageJ* (Abràmoff et al. 2004) to record male standard length (SL: snout tip to base of caudal fin). We then randomly assigned males to either an ablated ($n = 59$) or control ($n = 56$) group. While still under anesthetic, ablated males had the tip of their gonopodium removed with a scalpel, while control males underwent sham surgery (placed under dissecting microscope, gonopodium swung forward and then back into place without the ablation). Males were then transferred into individual 4-L tanks divided in half by a mesh barrier, and given three days to recover from the surgery, or sham surgery. In practice, males exhibited normal mating behaviors and feeding within minutes of recovery. To test for any differences in the rate of sperm priming of ablated and control males following exposure to visual and chemical cues from females, we first stripped males to empty their sperm reserves (Matthews et al. 1997) after the three-day recovery period. We then returned them to their individual tanks and introduced a non-pregnant, wild-caught female that was separated from the male by a mesh barrier (i.e., the male could see the female and perceive her odor cues) (Day 0). After four days, we again stripped the male and counted his total number of sperm (Day 4).

To test the second assumption about the effects of ablation on sperm release, we put newly stripped males (ablated: $n = 44$; control: $n = 42$) back into their individual tanks for another four days of exposure to the visual and chemical stimuli provided by a female. These were the same males used to test the first assumption, although some of them died prior to testing the second assumption. On Day 8, we removed the mesh barrier and

allowed the male and female to freely interact for 4 days. Subsequently, we stripped the male and counted his sperm (Day 12). We then compared the sperm counts of control and ablated males. During the 12-day experimental period, we rotated females between tanks daily to ensure that focal males remained sexually interested in the stimulus female (Vega-Trejo et al. 2014). Due to laboratory space limitations, this part of the experiment was run in two blocks (Block 1: $n = 22$ ablated, 25 intact males; Block 2: $n = 37$ ablated, 31 intact males). Overall, experimental males had a mean body size of 20.92 mm SL (± 0.09 SE). There was no significant difference in the average size of males in the ablated and control groups (GLM, $F_{1,111} = 0.296$, $P = 0.588$), or between experimental blocks (GLM, $F_{1,111} = 1.570$, $P = 0.213$), nor were there any significant differences between the two types of males over the two experimental blocks (block*male type interaction, GLM, $F_{1,110} = 0.102$, $P = 0.751$).

Experiment 2: female cues, male competitiveness and sperm priming response

To test for effects of female-derived cues on sperm priming, we created four treatments:

1. Naïve: an intact male housed alone ($n = 55$)
2. Chemical cues from a female: an intact male separated from a female by an opaque barrier that is permeable to water movement to allow chemical cues to reach the male (a preliminary dye test confirmed that water from both sides mixed well within four hours) ($n = 55$)
3. Chemical + visual cues from a female: intact or ablated males separated from a female by a mesh barrier. He can see the female but is unable to physically interact with her ($n = 68$)
4. Chemical + visual + behavioral cues from a female: an ablated male housed with full access to a female whom he can chase and harass, but is unable to release sperm ($n = 55$)

Based on the results of Experiment 1 (see “Results” below), we adopted the ablation surgery on the gonopodium tip to quantify potential increases in sperm available for mating in treatment 4. We measured males using the same methods described for Experiment 1 (mean \pm SE: 20.71 \pm 0.08 mm SL) and randomly assigned them to one of the four treatments. Male body length did not differ significantly among the treatments (ANOVA, $F_{3,204} = 2.571$, $P = 0.055$). Males were initially placed individually into one half of a 4-L aquarium for three days. To create treatments where males had different cues from females, we divided the aquarium in half using either an opaque, but chemically permeable, barrier (treatment 2), a mesh barrier (treatments 1 and 3), or no barrier (treatment 4). After three days, we stripped males to remove their sperm reserves (Day 0) and placed a wild-caught female

into the other half of the tank (for treatments 2 and 3) or with the male (treatment 4). Control (naïve) males were housed alone (treatment 1). We used size-matched, non-gravid females with an obvious anal black spot (Peden 1973) as social stimuli. We rotated the females among tanks daily to maintain male sexual interest. After four days, we stripped the males again and counted their total number of sperm (Day 4). Experiment 2 was set up at the same time as Block 2 of Experiment 1, meaning that all four treatments of Experiment 2 could be run concurrently (note: males from Block 2 of Experiment 1 constituted treatment 3 of Experiment 2). Treatment 3 therefore contained a mixture of ablated ($n = 37$) and intact ($n = 31$) males, but our results are robust to the use of only intact, or only ablated males (Supplementary Material).

Sperm count measurements

The methods used to collect and record the total sperm count for each male followed those described in O’dea et al. (2014), Head et al. (2015), and Vega-Trejo et al. (2019). In brief, after anesthetizing a male in ice water, we put him on a glass slide covered with 1% polyvinyl alcohol solution (PVA), swung his gonopodium forward, and gently pressed on his abdomen to eject sperm bundles (conducted by MHC for consistency) and added 100 μ l of extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl₂, 0.49 mM MgCl₂, 0.41 mM MgSO₄, 10 mM Tris (Cl); pH 7.5) to the ejaculate to prevent it from drying out. Using a 200- μ l pipette, we then transferred the sperm bundle solution to a 1.5-mL Eppendorf tube containing a further 500–800 μ l of extender medium (exact dilution dependent on ejaculate size to optimize the sperm count process). To break up sperm bundles and ensure the even distribution of sperm, we vortexed the solution for 20 s and mixed it repeatedly using a 10- μ l pipette. Next, 3 μ l of the sperm solution was placed onto a 20- μ m capillary slide (Leja) and the sperm counted using a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) under $\times 100$ magnification. For each male, we took five count measurements (mean number/sample: 19) from haphazardly selected points on the slide. Sperm counts were significantly repeatable ($r \pm$ SE = 0.862 \pm 0.011, $P < 0.001$, $N = 327$), and we therefore used the mean value, corrected for the initial dilution factor to determine each male’s total sperm count. To minimize observer bias, blinded methods were used when all sperm data were collected and analyzed.

Statistical analyses

In all analyses, sperm count data were log-transformed to meet assumptions of normality. To test for an effect of ablation surgery on sperm priming and sperm release by males in Experiment 1, we ran two separate general linear models: (1) to examine the

effect of ablation on Day 4 sperm count (priming response) and (2) to examine the effect on Day 12 sperm count (after mating interaction with females). Male type (ablated or sham surgery) and experimental block (two levels) were treated as fixed factors, with male body size as a fixed covariate (centered, i.e., mean of 0 and SD of 1) to account for a known positive correlation between male size and sperm number. To test for any effect of ablation surgery on the magnitude of the absolute change in sperm count between Days 4 and 12, we also ran a linear mixed model with total sperm count as the dependent variable, day (4 or 12), male type (ablated or intact) as fixed factors, and male identity as a random factor to account for two measures per male. We included the interaction between male type and day to check for potential differences in the magnitude and/or direction of the change in sperm count between the two types of males. We treated block as a fixed factor to control for absolute differences in sperm counts between experimental blocks revealed in our first set of analyses. We also treated male body size (standardized) as a fixed covariate in the model.

To test for the effect of different female mating cues on sperm priming, we ran a general linear model with sperm count as the dependent variable, treatment type (4 levels) as a fixed factor, and male body size (standardized) as a fixed covariate. We initially included the interaction between treatment and male body size in the model. If it was non-significant, we re-ran the model with it excluded and interpreted the main effects from the reduced model.

All results are presented as mean \pm SE, with analyses conducted in Rstudio v1.1.463 using R v3.5.2 (R Core Team 2018). We set the level for significance as $P \leq 0.05$ with two-tailed tests. The P values for linear mixed models were obtained using the ANOVA function of the ‘car’ package (type III Wald F-tests with Kenward-Roger degree of freedom) (Fox and Weisberg 2018) (R Core Team 2018). The statistical analyses for this study were pre-registered on the

Open Science Framework (OSF) in the Center for Open Science and can be viewed at <https://osf.io/2nu85>. As per our registration protocols, we excluded from our analyses one male in Experiment 1 with an abnormally high Day 4 sperm count (> 3 SD from mean value of all males). We also excluded one Experiment 2 male with an abnormally low sperm count as we were unable to rule out an error in data transcription. For completeness, we re-ran all analyses including these two males (see [Supplementary Material](#)) and confirmed that their inclusion did not change our main findings.

Results

Experiment 1: effect of gonopodium tip ablation surgery on sperm priming and release

Controlling for body size (GLM, $F_{1,110} = 11.28$, $P = 0.001$), there was no effect of removing the gonopodium tip on the sperm priming response of male *G. holbrooki*. Intact and ablated males had similar sperm counts after four days of exposure to visual and chemical cues from females (GLM, $F_{1,110} = 0.004$, $P = 0.948$) (Fig. 1a). Although absolute sperm counts differed between experimental blocks (GLM, $F_{1,110} = 38.91$, $P < 0.001$), the lower sperm counts recorded in Block 2 were equivalent for ablated and intact males (GLM, block*male type interaction, $F_{1,109} = 0.816$, $P = 0.368$).

Removal of the gonopodium tip had a significant effect on male sperm release (i.e., sperm count on Day 12 following full access to a female). Controlling for body size (GLM, $F_{1,81} = 5.788$, $P = 0.018$), ablated males had a significantly higher sperm count on Day 12 after interacting with females than did control males (GLM, $F_{1,81} = 25.829$, $P < 0.001$) (Fig. 1a). This suggests that contact between the gonopodium tip and the female’s gonopore or genital tract affects male sperm

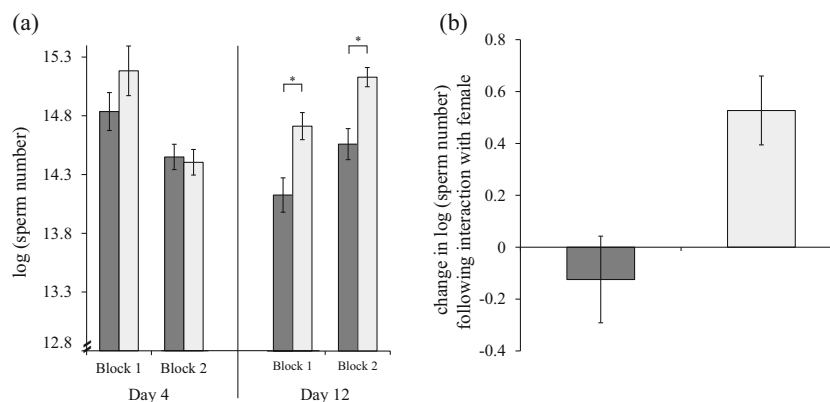


Fig. 1 **a** Mean \pm SE log-transformed sperm counts of males on Day 4 (following four days of visual and chemical cues from female, but no mating opportunity) and Day 12 (after full access to a female) in Experiment 1. **b** Difference of mean \pm SE log-transformed sperm counts (Day 12 minus Day 4) in each male. Gray = intact males, white = ablated

males. An asterisk above the error bars represent a significant difference between groups. The dataset for Day 4 only includes males who survived to Day 12 (Block 1: intact $n = 18$, ablated $n = 13$; Block 2: intact $n = 24$, ablated $n = 30$)

release. Again, there was a significant effect of experimental block on absolute Day 12 sperm counts (GLM, $F_{1,81} = 9.104$, $P = 0.003$), but no differential effect of male type across blocks (GLM, block*male type interaction, $F_{1,80} = 0.004$, $P = 0.950$). Controlling for body size (GLMM, $F_{1,81} = 6.783$, $P = 0.011$) and experimental block (GLMM, $F_{1,81} = 1.093$, $P = 0.299$), there was a significant interaction between day and male type on the change in sperm count between Days 4 and 12 (GLMM, $F_{1,83} = 10.959$, $P = 0.001$) (Fig. 1b). Intact (control) males showed a non-significant decrease in sperm count from Days 4 to 12 which included four days of mating contact with a female (paired t test, $t_{41} = 0.745$, $P = 0.460$), whereas ablated males with their gonopodium tip removed showed a significant increase in sperm number from Days 4 to 12, suggesting that they had not released sperm, despite attempting to mate with a female (paired t test, $t_{42} = -3.967$, $P < 0.001$).

Experiment 2: female cues, male competitiveness, and sperm priming response

Larger males replenished sperm at a significantly faster rate (GLM, $F_{1,203} = 25.763$, $P < 0.001$) (Fig. 2). This effect of male body size did not differ among the four treatments (GLM, male body size*treatment interaction: $F_{3,200} = 0.334$, $P = 0.801$). Although males who were exposed to the highest level of female mating cues (i.e., treatment 4: visual+ chemical +

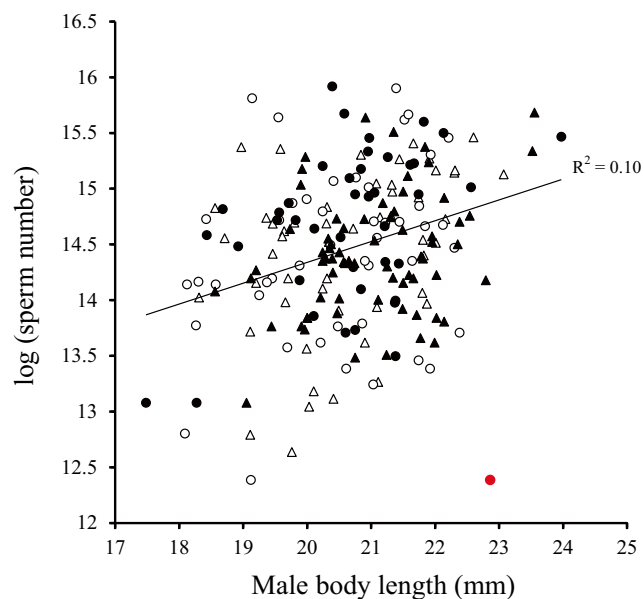


Fig. 2 Relationship between male body size and log-transformed sperm counts by male mosquitofish under either (1) no cue ($n = 53$; white, triangle), (2) chemical ($n = 46$; white, circle), (3) chemical + visual ($n = 68$; black, triangle), or (4) chemical + visual + behavioral ($n = 41$; black, circle) cues. A regression line is provided for the overall effect of body size (solid, black). Note: one large male from treatment 2 was excluded as an outlier (red, circle)

behavioral cues) had the slightly higher mean sperm count, the overall effect of access to female cues on the rate of sperm production did not differ significantly among the four treatments (GLM, $F_{3,203} = 2.451$, $P = 0.065$) (Fig. 3).

Discussion

Resource limitation and costly sperm production can favor the evolution of strategic investment into ejaculate traits by males (Wedell et al. 2002; Zbinden et al. 2003, 2004; Simmons et al. 2007). We therefore predicted that male mosquitofish (*Gambusia holbrooki*) would increase the available sperm reserves for mating if the number of cues indicative of female presence, hence mating opportunities, increased. We found, however, that males did not adjust the number of available sperm. There was, at best, a weak, non-significant trend for male with direct access to females to show higher levels of sperm priming. Male competitiveness, based on body size, affected baseline sperm count and sperm priming: larger males had more sperm and more rapidly replenished their sperm reserves. Nevertheless, there was no support for our prediction that male size moderates sperm priming based on the assumption that a trade-off between investment in pre- and post-copulatory mating traits would generate weaker sperm priming responses by males that have a greater advantage in pre-copulatory sexual selection due to their larger body size (Pilastro et al. 1997).

Why no sperm priming in *G. holbrooki*?

The lack of detectable male sperm priming in *G. holbrooki* in response to cues of female availability contrasts with results in other Poeciliids, like guppies (Cattelan and Pilastro 2018) and

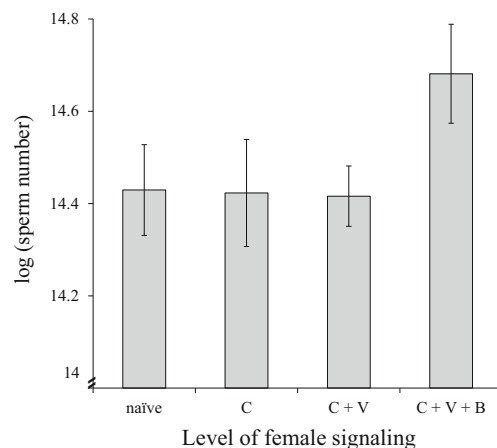


Fig. 3 Effects of varying levels of signals from females on sperm priming response. Four levels of female signaling are presented: (1) no cue (naïve; $n = 53$), (2) chemical (C; $n = 46$), (3) chemical + visual (C + V; $n = 68$), (4) chemical + visual + behavioral (C + V + B; $n = 41$) cues. Mean \pm SE

sailfin mollies (Aspbury and Gabor 2004). Studies of both of these species reported similar effect sizes, and detected sperm priming with modest sample numbers [guppies: $\eta^2 = 0.257$, $n = 10$ (Bozynski and Liley 2003); mollies: $\eta^2 = 0.215$, $n = 18$ (Aspbury and Gabor 2004)]. Given an equivalent effect size in mosquitofish, and 55 males/treatment, we had >80% power to detect a significant difference among groups ($n = 27$ /group to detect an effect size of 0.257 with 80% power; see Cohen 1988). We observed an effect size in mosquitofish of $\eta^2 = 0.023$, which is suggestive of a biological difference between *G. holbrooki* and other Poeciliid species in the benefits of elevating the number of available sperm. We offer two explanations for the difference based on the mating system, and the potential for sex-specific cues to affect sperm priming.

First, both guppies and mosquitofish are polyandrous, such that males experience sperm competition, but only guppies have alternative mating tactics. Most research to date on plasticity in sperm priming response has focused on species like guppies and mollies that both court and mate coercively. Bozynski and Liley (2003) demonstrated that male guppies increase the number of available sperm upon sighting a female, but subsequent studies have further showed that the higher rate of sperm production is associated with a switch from courtship to coercive mating (Devigili et al. 2015; Cattelan et al. 2016). In guppies, female receptivity affects male reproductive success (Evans et al. 2002; Pilastro et al. 2002, 2004; Guevara-Fiore and Endler 2018). Courtship displays increase male mating success more than “sneak” mating attempts, but they are energetically far more costly (Matthews et al. 1997; Pilastro and Bisazza 1999; Evans and Magurran 2001). Investment in “persuading” females to mate thus limits the energy available for sperm allocation by courting males. A trade-off between the dominant mating tactic and spermatogenesis therefore creates the potential for adaptive plasticity of sperm priming to evolve in response to female availability (Gasparini et al. 2009; Cattelan and Pilastro 2018). In contrast, mosquitofish have a single coercive mating tactic (Pilastro et al. 2003; Dadda et al. 2005), with a weak or no correlation between a male’s maximal sperm reserves and how many sperm are successfully transferred to a female (Head et al. 2015). Male reproductive success is more likely to depend on the rate of coercive mating attempts than the number of available sperm (Deaton 2008). The marginal benefits of sperm priming are therefore likely to be weak for male mosquitofish. Further support for this argument is that in guppies, a single ejaculation can reduce stored sperm by up to 92% (Pilastro and Bisazza 1999), while we found a much lower risk of sperm depletion in mosquitofish in our study. Intact males who had constant access to females for four days had, on average, only an 8.6% decline in sperm reserves compared to their maximal reserve level. Finally, it is also possible that the less costly coercive mating tactic (compared to courtship displays in guppies; Cattelan et al. 2016) does not constrain investment in sperm, such that male mosquitofish can maintain a relatively steady amount of sperm

reserves even when they make more copulation attempts due to more mating opportunities.

Second, the discrepancy between our findings and those of other studies might relate to a difference in the mating opportunity cues provided by male competitors versus females. Males often use cues from competitors to evaluate relative female availability and the likely levels of sperm competition (Wedell et al. 2002; Pizzari et al. 2003; Zbinden et al. 2003, 2004; Lüpold et al. 2011). Previous studies of sperm priming in Poeciliids have often varied the number and/or presence of competitors (Kelly and Jennions 2011). The variation in female cues detected by the focal males in our study might be a weak predictor of mating opportunities in the wild (Jordan and Brooks 2012; Kahn et al. 2013). Bisazza and Marin (1995) noted that insemination success decreases as the number of rivals increases, so cues about the number of rivals are potentially a better predictor of mating opportunities than any cues provided by females (Bisazza and Marin 1995; Bisazza et al. 1996; Harrison et al. 2018). Male mosquitofish might gain more from strategically adjusting sperm production in response to male-derived cues about sperm competition (Evans et al. 2003) than from female-derived cues. Indeed, tactic-dependent plasticity in sperm velocity in response to cues from male competitor has been reported in *Xiphophorus nigrensis* (Smith and Ryan 2011). An environment where rivals are absent, as in our study, might therefore fail to trigger sperm priming (Bretman et al. 2011).

We found evidence for condition-dependent sperm priming response in male mosquitofish: larger males had higher sperm reserves and faster sperm replenishment. Our prediction that smaller males would compensate with a stronger sperm priming response has empirical support from studies in other species (Aspbury 2007; Tamara Montrose et al. 2008; Devigili et al. 2015). There was, however, no effect of relative competitiveness (i.e., body size) on sperm priming in *G. holbrooki*. This might be because larger male mosquitofish cannot fully monopolize females or prevent smaller males mating (Bisazza and Marin 1995; Pilastro et al. 1997; McCullough et al. 2018). This serves to reduce size-based variation in mating success and sperm competition risk (Simmons et al. 1999; Kelly 2008). The observed covariation between body size and sperm number is therefore more likely to be driven by condition-dependent spermatogenesis than strategic adjustment of investment into pre- and post-copulatory traits (Locatello et al. 2006; Hill 2011; Lüpold et al. 2014).

A method to manipulate sperm production

A few studies have examined the function of the gonopodium tip for insemination success (Peden 1972; Kwan et al. 2013) but they were unable to disentangle the causes of failed sperm transfer, in which both sperm release and accurate sperm deposition might play a role. We documented, perhaps for the first time, the role

of the gonopodium tip in sperm release by male mosquitofish. Males without a gonopodium tip showed a significant increase in sperm count over the four days they spent with a female, suggesting that they did not release sperm. We also confirmed that removal of the gonopodium tip did not affect the rate of sperm replenishment or sperm priming. That is, ablated and intact males exposed to the same female-derived cues had equivalent sperm counts four days after being stripped (Experiment 1). Our results agree with those of Evans et al. (2003) in showing no significant effect of exposure to different levels of perceived sperm competition risk on sperm priming, but our study has the advantage that we controlled for sperm release during the stimulus period. We can therefore rule out that the lack of observed differences in sperm priming among our treatments was due to changes in the rate of ejaculation and/or ejaculate size. Our study is therefore the first to test the independent effect of full access to a female on rates of sperm production (i.e., by preventing sperm release), when most studies typically only focus on how visual cues affect reproductive strategies in Poeciliid fishes (e.g., Kodama et al. 2008; Auld et al. 2017). Ablation surgery could become a valuable tool to disentangle the effects of sperm reserve depletion, ejaculate investment, and mating effort on male mating success and longevity (Bisazza et al. 1996; Parker et al. 2013; Devigili et al. 2015; Iglesias-Carrasco et al. 2019).

Our finding that the gonopodial tip is essential for sperm release in mosquitofish should prompt enquiry about the precise mechanism of sperm release, which is currently unknown. The copulatory process lasts less than 900 ms in *Gambusia* (Warburton et al. 1957 cited by Pyke 2005), but the duration of insertion is far shorter and difficult to see, even with high-speed camera footage (personal observation). Previous studies assume that the twisting of the ligament at the base of the fin during the forward swing of the gonopodium is the mechanism by which sperm bundles are transferred from the testes to the gonopodium tip (Collier 1936; Rosen and Tucker 1961). Our results suggest, however, that the forward-swinging motion of the gonopodium is insufficient to lead to sperm release: ablated males could swing their gonopodium forward, but failed to release sperm. Instead, we suggest there is a role for sensory or mechanoreceptors at the tip that allow for precise control over the timing of ejaculation. Further investigation of the role of the various hooks and spines on the gonopodium tip in allowing males to determine when contact has been made with a female's gonopore offer an interesting avenue for future research.

Conclusion

We did not find phenotypic plasticity in sperm priming in mosquitofish in response to cues of female presence, nor any moderating effect of male size on the rate of sperm production in response to different numbers of cues. We did, however,

provide experimental confirmation that ablation of the gonopodial tip of male mosquitofish has no effect on their sperm replenishment rates; and that ablated males do not release sperm when housed with a female. This suggests that the gonopodium tip is involved in triggering sperm release. We suggest that ablation surgery is a valuable technique to use to ask questions about male reproductive costs (e.g., costs of sperm production versus mating effort).

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Author contributions All authors contributed to the study conception and design. MHC collected and analyzed the data and wrote the first draft of the manuscript. All authors contributed to subsequent revisions and approved the final manuscript.

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Data availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable national and institutional guidelines for the use of animals were followed. Fish were collected under a Scientific License from the Australian Capital Territory (ACT) Government, granted under Section 21 of the Fisheries Act 2000 (license no. FS20188). All experimental procedures were performed in accordance with approvals granted by the ANU Animal Ethics Committee (approval no. 2018/27).

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