




An experimental test for body size-dependent effects of male harassment and an elevated copulation rate on female lifetime fecundity and offspring performance

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Abstract

Many studies investigate the benefits of polyandry, but repeated interactions with males can lower female reproductive success. Interacting with males might even decrease offspring performance if it reduces a female's ability to transfer maternal resources. Male presence can be detrimental for females in two ways: by forcing females to mate at a higher rate and through costs associated with resisting male mating attempts. Teasing apart the relative costs of elevated mating rates from those of greater male harassment is critical to understand the evolution of mating strategies. Furthermore, it is important to test whether a male's phenotype, notably body size, has differential effects on female reproductive success versus the performance of offspring, and whether this is due to male body size affecting the costs of harassment or the actual mating rate. In the eastern mosquitofish *Gambusia holbrooki*, males vary greatly in body size and continually attempt to inseminate females. We experimentally manipulated male presence (i.e., harassment), male body size and whether males could copulate. Exposure to males had strong detrimental effects on female reproductive output, growth and immune response, independent of male size or whether males could copulate. In contrast, there was a little evidence of a cross-generational effect of male harassment or mating rate on offspring performance. Our results suggest that females housed with males pay direct costs due to reduced condition and offspring production and that these costs are not a consequence of increased mating rates. Furthermore, exposure to males does not affect offspring reproductive traits.

KEYWORDS

body size, cross-generational effects, fitness, *Gambusia holbrooki*, harassment, sexual conflict

1 | INTRODUCTION

When males provide material benefits to females, such as nuptial gifts or access to food, multiple mating can increase a female's lifetime reproductive success by elevating her fecundity

per breeding attempt or by extending her reproductive lifespan (Chapman, Miyatake, Smith, & Partridge, 1998; Johnstone & Keller, 2000; Møller & Jennions, 2001). However, repeated interactions with males can also lower a female's lifetime reproductive success (Arnqvist & Nilsson, 2000; Arnqvist & Rowe, 2005). The sexes differ

in the fitness returns from additional mating, which often leads to the evolution of female mating resistance and repeated mating attempts by coercive males (i.e., "male harassment"). The net result of this sexual conflict can be a decline in female fitness due to detrimental effects of elevated copulation rates and/or because resisting or evading male mating attempts is costly. The costs of copulation to females can include an increased risk of predation (Darden & Croft, 2008) or injury while mating (Blanckenhorn *et al.*, 2002; Crudgington & Siva-Jothy, 2000), and the acquisition of sexually transmitted diseases (Ashby & Gupta, 2013), or of toxins and other harmful compounds that are transferred in seminal fluid (Chapman, 2001; Chapman, Liddle, Kalb, Wolfner, & Partridge, 1995). To avoid these costs of copulation, females have evolved to resist or evade male mating attempts, but rejecting males can be costly (Harano, 2015). Specifically, the time spent avoiding male harassment can lower feeding opportunities (Griffiths, 1996; Pilastro, Benetton, & Bisazza, 2003), and mate rejection can increase the risk of being injured by males (Adler, 2009). Many studies have now shown that the greater presence of males (e.g. more male-biased operational sex ratios) is associated with decreased female longevity and/or lower fecundity (Rönn, Katvala, & Arnqvist, 2006; Takahashi & Watanabe, 2010; Iglesias-Carrasco, Bilgin, Jennions, & Head, 2018, but see Head & Brooks, 2006). In general, experimental studies show that females continuously housed with males have lower fitness than females that only have intermittent access to males (Edvardsson, 2007; Lew, Morrow, & Rice, 2006; Rönn *et al.*, 2006). In most of these studies, however, researchers cannot determine the relative effects of the actual rate of copulation from those of male sexual harassment: the increased presence of males leads to both greater male harassment and a higher mating rate (but see Fox, Head, & Jennions, 2019; den Hollander & Gwynne, 2009; Partridge & Fowler, 1990; Zajitschek, Dowling, Head, Rodriguez-Exposito, & Garcia-Gonzalez, 2018). The distinction between these two processes matters. If we aim to understand the evolution of female mating strategies and behaviours, it is necessary to tease apart how the various costs of higher mating rates (with one or with several males) versus male harassment influence net female fitness.

Male harassment and elevated mating rates might also lower the reproductive value of a female's offspring if they reduce beneficial maternal effects. For example, females might allocate fewer resources to eggs or decrease parental care to ameliorate costs of repeated copulation or greater male harassment. To date, the effects of increased male presence on offspring fitness have rarely been quantified (Brommer, Fricke, Edward, & Chapman, 2012; Le Galliard, Cote, & Fitze, 2008; Priest, Galloway, & Roach, 2008; Zajitschek *et al.*, 2018) and studies have found conflicting results, even within a species. For example, Dowling, Williams, and Garcia-Gonzalez (2014) showed that female *Drosophila melanogaster* experiencing more sexual interactions produced offspring with lower survival and higher rates of senescence, whereas Priest *et al.* (2008) found that females that mated more often produced daughters with greater lifetime reproductive success. As with attempts to investigate the effects of male presence on female fitness, the relative importance

of greater male harassment versus elevated mating rates for determining offspring fitness is rarely known. For example, in guppies, *Poecilia reticulata*, females exposed to greater male harassment produced smaller daughters and sons with shorter gonopodia, lowering their reproductive success, but these females also had a higher mating rate (Gasparini, Devigili, & Pilastro, 2012). Likewise, few studies have determined the relative effect of male harassment and mating rate on female fitness versus offspring fitness. For example, Gasparini *et al.* (2012) showed that male presence affected offspring but did not affect the mother's fecundity, but it is unknown whether this was due to differential within- and cross-generational effects of male harassment, the actual mating rate or both.

The costs and benefits of avoiding or accepting mating attempts are likely to depend on a male's phenotype. For example, females often prefer males that provide greater material benefits or greater protection (Bierbach, Sassmannshausen, Streit, Arias-Rodriguez, & Plath, 2013; Møller & Jennions, 2001), or sire offspring of above-average fitness (Firman, Gasparini, Manier, & Pizzari, 2017; Hosken, Taylor, Hoyle, Higgins, & Wedell, 2008). In several cases, these high-quality males are larger than average (Arnqvist & Rowe, 2005; Charlton, Reby, & McComb, 2007; Lehmann & Lehmann, 2008). However, male body size is a trait that is likely to affect not only the benefits but also the costs of interacting with males. For example, in some species females prefer to mate with larger males that impose greater costs on females, reducing female lifespan and net fitness (Friberg & Arnqvist, 2003; Pitnick & Garcia-Gonzalez, 2002). In contrast, in other species where small males tend to behave more sneakily and engage in increase harassment to gain access to females (Schlupp, Knab, & Ryan, 2001), females can benefit from choosing to associate with large males, if they provide protection and direct benefits during copulation. To date, the effects of male body size have mainly been investigated by asking whether larger males produce fitter offspring (e.g. Mainguy, Coté, Festa-bianchet, & Coltman, 2009; Røed *et al.*, 2007). Fewer studies test whether the extent of the effect that copulations and harassment have on offspring traits varies with male size. This is an oversight: greater understanding of cross-generational effects of body size might help to explain the maintenance of high variation in body size in many wild populations.

The eastern mosquitofish, *Gambusia holbrooki* Girard, 1859 is an ideal species to test for male body size-dependent costs of mating and/or male harassment on the fitness of females and their offspring. It is a livebearing poeciliid with internal fertilization. Males rarely court, instead incessantly pursue females and try to forcibly copulate (Bisazza & Marin, 1991). Males make frequent copulation attempts (up to one attempt/minute, Wilson, 2005) by approaching females from behind and thrusting their gonopodium (a modified anal fin used to transfer sperm) towards the female's gonopore. Females often attempt to either evade or attack males, which is likely to be energetically costly. In addition, gonopodia have spines on the tip that sometimes damage females and cause oviducal bleeding (Sommer-Trembo, Plath, Gismann, Helfrich, & Bierbach, 2017). Male harassment and the act of copulation are likely to impose cumulative costs on females. Although males do not provide females with material benefits

(i.e. no nuptial gifts), females mate multiply (Head, Kahn, Henshaw, Keogh, & Jennions, 2017; Zane, Nelson, Jones, & Avise, 1999), and recent evidence suggests that polyandry can both elevate female reproductive success and alter the phenotype of their offspring (Fox *et al.*, 2019). In wild populations, adult male body size is highly variable despite minimal post-maturation growth (Kahn, Mautz, & Jennions, 2010). In general, larger males have higher insemination success per mating attempt (Head, Vega-Trejo, Jacomb, & Jennions, 2015, but see Pilastro, Giacomello, & Bisazza, 1997) and females prefer to associate with larger males (Bisazza, Vaccari, & Pilastro, 2001; Kahn *et al.*, 2010). This might indicate that females associate and copulate with larger males because they impose lower costs than smaller males which harass, and attempt to copulate with, females more (Bisazza & Marin, 1995; Hughes, 1985; Pilastro *et al.*, 1997).

Here we tease apart the effects of male harassment and the actual mating rate by using males that either can or cannot copulate on the lifetime reproductive success of females and the performance of their offspring. We also explore the effects of continual exposure to either small or large males, or to no males. To do this, we housed female *G. holbrooki* for their natural reproductive lifespan (23 weeks) with either a small male, a large male or no male (control). All females were artificially inseminated with sperm of the corresponding male size (including the controls) so that even females housed without males could still breed. To disentangle the effects of harassment and copulation, males either had an intact gonopodium or had the tip of their gonopodium surgically removed (ablated). Both intact and ablated males can harass females and try to mate, but ablated males cannot copulate (Kahn *et al.*, 2010; Mautz, 2011). We measured female fecundity, as well as female adult growth rate and immune response as proxies for the relative costs imposed by males that differ in size and ability to copulate. To test for any cross-generational effects, we then reared offspring from each female and measured performance-related traits such as size at birth, juvenile growth rate and the mating performance of sons (see Figure 1 for diagram on the background of the hypothesis tested).

2 | MATERIALS AND METHODS

2.1 | Origin and maintenance of individuals

We collected mature mosquitofish males and recently matured females in Canberra in November–December 2017 at the start of the breeding season. We placed 300 recently matured females into single-sex 90-L aquaria (50 fish/tank), under a 14:10-hr light:dark photoperiod at 28°C ($\pm 1^\circ\text{C}$). Females were kept in these tanks until we had captured sufficient males to set up our experiment. On the day of collection, we anaesthetized males with a brief immersion in iced water, and measured their body size using dial callipers. We created two size classes within the pool of available males (size ranging from 18.6 mm to 28.00mm): small (S) ≤ 21 mm (mean 19.1 ± 0.06 mm SE) and large (L) ≥ 24 mm (mean 24.6 ± 0.05 mm SE). Upon maturation, males almost completely stop growing (Vega-Trejo, Fox, Iglesias-Carrasco, Head, & Jennions, 2019), ensuring that the magnitude of

the size treatment remained similar during the whole experiment. Males were housed in size-specific 90-L aquaria in groups of 50 until we had 200 males of each size class. Half the males in each size class were then anaesthetized in iced water, and we surgically removed the gonopodium tip under a dissecting microscope (Kahn *et al.*, 2010). We created four types of male: large intact (Li), large ablated (La), small intact (Si) and small ablated (Sa). Ablated males can harass, but cannot copulate, with females (i.e. they do not transfer sperm because they cannot insert their gonopodium into the oviduct). Males were returned to their single-sex tanks for 3 days to recover from the procedure. Survival was 100%, and males exhibited normal behaviour within minutes of the ablation procedure. Fish were fed ad libitum twice daily with *Artemia salina* nauplii and commercial fish flakes.

2.2 | Experimental design

To investigate how male harassment, the ability of males to copulate and male body size influence female fitness and offspring performance, we used a 3x2 factorial experimental design (Figure 2). Females were either housed with a small male (S), a large male (L) or no male (control, c, but inseminated with sperm of males of the corresponding size), and the male was either ablated (a) or intact (i). In all cases, females were artificially inseminated (AI) with sperm from males of the same size class as the males with whom they were housed, and control females were inseminated with sperm from either large or small males. Details of the AI protocol are given below. This created six treatments: Lc, Sc, Li, Si, La and Sa ($n = 50$ replicates per treatment). The use of Lc and Sc males allows us to control for any genetic differences in the sperm of large and small males that might affect offspring phenotypes when considering the effects of male presence and copulation rate on offspring traits.

Females were housed either alone or with a male in a 4-L tank with artificial plants for refuge. There was a < 5 mm mesh barrier

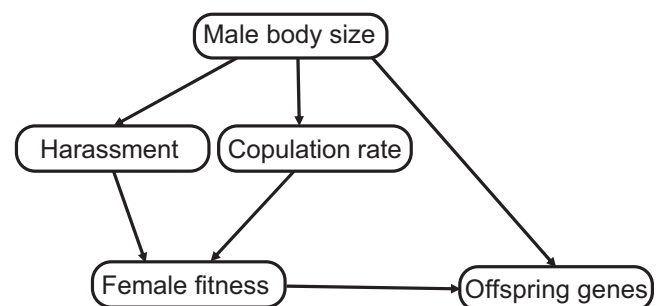


FIGURE 1 Diagram explaining the theoretical background of the experiment. Male body size is expected to affect male mating behaviour, such as the intensity of harassment, as well as the copulation rate if males that differ in body size vary in their mating success. Changes in both these male characteristics can affect the costs associated with male exposure for females, hence detrimentally affecting their fitness. Finally, offspring genes and traits are expected to be affected by male size if any trait associated with body size is heritable, as well as by females through any maternal effect derived from female exposure to males

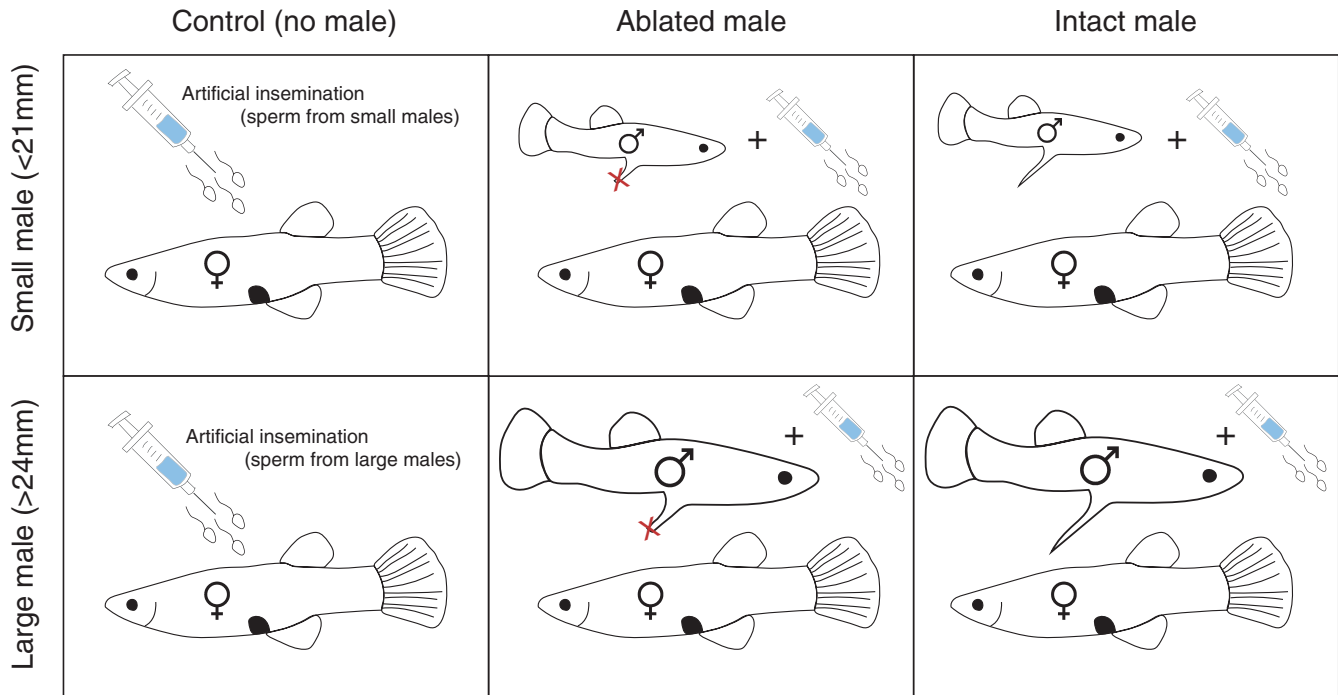


FIGURE 2 Experimental design. Females were exposed to one of the three male status treatments: control, no male but inseminated with the corresponding sperm of small or large males; ablated male able to harass but not copulate (tip of gonopodium surgically removed); intact male able to harass and copulate. At the same time, males differed in their body size (large vs. small). Additionally, females exposed to both ablated and intact males were periodically inseminated with sperm of males of the corresponding body size. We set up 50 replicates in each treatment

enclosure at the front of the tank to create a refuge for any newborn offspring. Males within each treatment type were rotated between tanks weekly to maintain their sexual interest in the female and ensure ongoing copulation attempts. Females were artificially inseminated with sperm stripped from males of the corresponding size class each time within 72 hr of giving birth, or after 7 weeks had elapsed without giving birth, whichever was earlier. Although females might have not been receptive immediately after giving birth (in the wild, the time from birth of one brood and fertilization of the next can be anything from 2 days to 2 weeks; Pyke, 2005), female mosquitofish retain viable sperm in their oviduct for several months, giving them total flexibility over the timing of fertilization (Pyke, 2005). This meant that the timing of insemination, and its relationship to female receptivity, was not a factor in determining the relative likelihood of fertilization. In fact, the sperm storage ability of females ensures the production of offspring even when females are artificially inseminated during the nonbreeding period. We inseminated all females irrespective of whether or not they were housed with a male to control for effects of handling associated with AI. To inseminate a female, we randomly picked three males from a separate stock of wild-caught males of the appropriate size class. We anaesthetized a male in iced water, placed him on a glass slide under a dissecting microscope, swung his gonopodium forward and gently pressed his abdomen to eject his sperm. We then pipetted 100 μ l of saline solution (0.9% NaCl) onto the slide and transferred the sperm solution to an Eppendorf tube. The process was repeated for the

other two males, and their sperm transferred to the same Eppendorf tube (Head *et al.*, 2015). We allowed sperm to settle together at the bottom of the tube for several minutes, and then, we used a micropipette to transfer 3 μ l of the sperm mixture into the oviduct of the anaesthetized female and returned her to her treatment tank. This method has been used to artificially inseminate females before with a high success rate (Fox *et al.*, 2019). We kept females in the treatments for 23 weeks, which is the approximate length of the *Gambusia* breeding season in our study population (Kahn, Kokko, & Jennions, 2013). Finally, all fish were euthanized as legislation outlaws the release of pest species. All experimental procedures were carried out under approval from The Australian National University Animal Ethics Committee (Approvals A2015/07 and A2018/27) and complied with existing laws regulating the treatment of vertebrates in Australia.

All the measurements described below were collected blind to the female's treatment.

2.3 | Female fecundity, growth and immune response

2.3.1 | Female growth and lifetime reproductive success

We collected females at the start of the breeding season. All females were likely to have been naturally inseminated, and most

were obviously pregnant based on their belly size. However, to ensure females were inseminated, we initially placed nonpregnant females with several randomly chosen males for a week. Males were removed from the tanks, and 4 days later, we set up females in their corresponding treatments. We then excluded the first brood produced from our analysis because our experimental treatment is least likely to affect this brood. The number of broods excluding the first one produced by females during the 23 weeks of experiment ranged from 0 to 3 (mean \pm SE = 0.968 \pm 0.883). We checked experimental tanks twice daily for fry, and recorded the date of birth and the number of offspring. Female standard length (SL: snout tip to base of caudal fin) was recorded at the start and end of the experiment. We photographed the anaesthetized female alongside a 0.1 mm scale bar and then made measurements using *ImageJ* (Abràmoff, Magalhães, & Ram, 2004). Growth was calculated as the change in size from the start to the end of the experiment. There was no difference in the initial mean SL of females in each treatment ($F_{5,294} = 0.422$, $p = .833$).

2.3.2 | Female immune response

After 18 weeks, we measured the cell-mediated immunity of surviving females ($n = 275$ of 300) using a phytohaemagglutinin injection assay (PHA test). This assay has been used in other fishes (Clotfelter, Ardia, & McGraw, 2007) and been validated in *G. holbrooki* (Iglesias-Carrasco, Fox, Vincent, Head, & Jennions, 2019). We anaesthetized females in iced water and measured their caudal peduncle thickness at the posterior end of the dorsal fin with a pressure-sensitive spessimeter (Mitutoyo 547-301, accuracy: 0.01 mm; average of 5 measurements per fish). We then injected 0.01 mg of PHA dissolved in 0.01 ml of PBS into the left side of the caudal peduncle. Females were returned to their individual treatment tank for 24 hr, but housed alone, to eliminate any immediate effect of male presence. After 24 hr, we re-measured the peduncle thickness to calculate inflammation (difference between pre- and post-injection measures). Any tissue swelling disappeared within 72 hr.

2.4 | Maternal effects on offspring

2.4.1 | Offspring size at birth, growth rate, survival and sex ratio

To test for differences in the effects of harassment or being repeatedly mated by small or large males, we measured traits of offspring from each females' second brood. Offspring were removed from the experimental tank on the day of birth. Six fry per brood, unless fewer were produced, were then randomly selected ($n = 457$ offspring from 137 females: 140 Lc fry from 34 females, 144 Sc fry from 37 females, 36 La fry from 13 females, 45 Sa fry from 17 females, 40 Li fry from 16 females and 52 Si fry from 20 females). Newborn fry were placed in a small 1x1 cm container of shallow water over a 0.1-mm grid and photographed from above for later measurement in *ImageJ*. These offspring were then housed individually in 1-L tanks, on a 14:10-hr light:dark

photoperiod at 28°C, and fed twice daily with *Artemia salina* nauplii. We re-measured each juvenile at 3 weeks of age ($L_{\text{day}21}$) to calculate their daily growth $\left[\text{mm/day} \frac{L_{\text{day}21} - L_{\text{day}1}}{21} \right]$. At that stage, we also determined offspring survival (1:alive/0:dead). From 4 weeks of age, juveniles were inspected three times per week until we could determine their sex. Sons were retained to measure their reproductive performance (see below), and daughters were euthanized.

2.4.2 | Sons' reproductive quality

In total, 196 sons (58 Lc, 67 Sc, 10 La, 19 Sa, 21 Li and 21 Si) were tested to measure their reproductive performance. We measured seven traits previously linked to male reproductive success (e.g., Head *et al.*, 2017; Head *et al.*, 2015): (a) time to maturity, (b) body size at maturity, (c) relative gonopodium length, (d) attractiveness to females, (e) sexual behaviour, (f) sperm number and (g) sperm velocity.

From 4 weeks of age, sons were checked three times a week to determine their date of maturation (defined by a pointed gonopodium with clear distal spines). At maturity, we photographed each male after placing him laterally on his side with the gonopodium extended to measure his SL and gonopodium length (from tip to base along the leading edge). For statistical analyses, we used the relative gonopodium length, calculated as the residuals of the log-log regression of gonopodium length on body length.

At 6 weeks post-maturation, which is the approximate age at which male sperm traits reach their maximum values (Vega-Trejo *et al.*, 2019), we assayed each son's sexual attractiveness and mating behaviour. Attractiveness was measured in a two-choice tank in which a virgin stock female was presented with a focal son and a random competitor from the laboratory stock. The competitors were all 21–23 mm in size, which is the modal range in the study population (Kahn *et al.*, 2013). The two-choice tank had three compartments: a central one (49 × 20 × 22 cm) for the test female, and two end sections for males (each 7 × 20 × 22 cm). The focal son was randomly placed at one end and the competitor at the other. The external tank walls were covered in black plastic to minimize external distraction. Males were initially placed behind a mesh divider and an opaque screen to separate them from the female in the central compartment. After 10 min of acclimation, we removed the opaque screens, and for 10 min, we then recorded how much time the female spent in the association zone of each male (<5 cm from the mesh barrier). Trials in which the female did not visit the association zone of both males were discarded ($n = 15$ of 184). Stock males and virgin females were used in only one trial per day.

Following the attractiveness assay, sons were transferred to a 4-L tank containing a female behind a mesh barrier. The female was selected at random from a laboratory-maintained stock of 50 average-sized individuals (550–750 mg mass range) and placed in the tank >1 hr prior to the start of observations. Following a 5-min acclimation period for the male, the mesh partition was removed. For the next 10 min, we then recorded: (a) time spent by the male

chasing or associating with the female (<1 body length and oriented towards her); (b) number of mating attempts (gonopodial thrusts towards her gonopore after being positioned behind her); and (c) number of successful attempts (thrusts that contacted the gonopore).

Next, sons were returned to their individual 1-L tanks for 7 days to allow them to replenish their sperm reserves (O'Dea, Jennions, & Head, 2014). We then quantified two ejaculate traits: sperm number and velocity. For sperm collection and measurement, we followed the methods of Vega-Trejo, Jennions, and Head (2016). Briefly, we stripped sperm and collected two subsamples each containing three sperm bundles. The three bundles were each pipetted into an Eppendorf tube containing 2 μ l of extender medium (pH 7.5 with composition: 207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl₂, 0.49 mM MgCl₂, 0.41 mM MgSO₄, 10 mM Tris [Cl]) and retained for sperm velocity analyses. The rest of the ejaculate was pipetted and transferred to a 1.5-ml Eppendorf tube containing 600–1,000 μ l of extender medium. The amount of medium was adjusted to ensure the intermediate sperm concentrations that are required for accurate sperm counts. To estimate the number of sperm, we vortexed the solution for 1 min to break up sperm bundles and distribute sperm evenly throughout the sample. We then pipetted 3 μ l onto a 20- μ m capillary slide (Leja) and counted the number of sperm using a CEROS Sperm Tracker (Hamilton Thorne Research) under 100 \times magnification. We counted five subsamples per sample and estimated count repeatability using the rptR package (Nakagawa & Schielzeth, 2010). Since repeatability was high ($r = .897 \pm .011$ SE, $p < .001$), the mean value was used for further analyses. We corrected the total sperm counts for the six bundles that were removed to estimate sperm velocity. The number of sperm per bundle does not vary significantly across males so we used the mean value (7,677 \pm 477 SE sperm, $n = 50$ males, unpublished data). The threshold values to define cell detection were set as elongation percentage 15–65 and head size 5–15 μ m, and the static tail filter was set off.

To measure sperm velocity, we placed each of the two 2 μ l samples in the centre of a different cell of a 12-cell multi-test slide (MP Biomedicals) previously coated with 1% PVA solution to prevent sperm sticking to the slide. Each sample was then activated with a 3 μ l solution of 125 mM KCl and 2 mg/ml bovine serum albumin (Billard & Cosson, 1992) and a coverslip put in place. We analysed sperm velocity within 30 s of activation for an average of 40.54 \pm 1.55 SE sperm tracks per ejaculate (minimum 10 tracks/male). We recorded (a) average path velocity, VAP (the average velocity over a smoothed cell path), and (b) curvilinear velocity, VCL (the actual velocity along the trajectory) using a CEROS Sperm Tracker. The threshold values defining static cells were predetermined at 20 μ m/s for VAP and 15 μ m/s for VCL. VAP and VCL were strongly correlated ($r = .92$, $p < .001$) so we used the more biologically relevant measure of VCL in our analyses (Boschetto, Gasparini, & Pilastro, 2011). Given its significant repeatability ($r = .429 \pm .066$ SE, $p < .001$), we used the mean value in our analyses.

2.5 | Statistical analysis

All analyses were conducted in R 3.2.2 (R Core Team, 2015). To test whether the presence of a male, male body size and the ability to copulate affect female and offspring traits, we ran linear mixed models and generalized linear mixed models (LMMs or GLMMs) using the lme4 package. Male size (large or small) and male status (absent, ablated or intact) and their interaction were included as fixed factors in all models. We can use this 2 \times 3 interaction because even control females (male absent) were inseminated with sperm of small or large males. If the interaction was nonsignificant, we re-ran the model without it. If its removal did not significantly reduce the model fit, we interpret the main effects from the reduced model. We ensured the fit of models that assume a Gaussian error distribution by checking the distribution of residuals. When necessary, data were transformed using the log, square root or powerTransform function in the car package. When necessary (in the case of female fecundity and number of gonopodial thrusts), we also corrected for overdispersion in the models with a Poisson error distribution by specifying a randomly assigned identification number as a random effect (i.e., observation-level random effect; Harrison, 2014). After this correction, the dispersion parameter of our model was reduced from 3.45 to 0.317. None of the binomial models were overdispersed. We tested the significance of model terms using the ANOVA function of the car package, with the type III Wald chi-square tests (see Supplementary Information for test parameters). Finally, where relevant, we conducted post hoc pairwise comparisons using Tukey's tests.

2.5.1 | Female traits

We ran separate linear models and generalized linear models to test the effects of male status and body size on: (a) the proportion of females that bred again after the first brood (excluding the 55 that never gave birth and 25 that died, $n = 220$, binomial error, yes—gave birth to at least a second brood, $n = 140$ /no—never gave birth again, $n = 80$); (b) fecundity (for females that gave birth at least two broods, $n = 134$, Poisson error); (c) lifetime growth ($n = 274$, Gaussian error, power transformed); and (d) immune response ($n = 275$, Gaussian error). In models 1 and 2, initial female SL was included as covariate, whereas in model 4, final female SL was treated as a covariate. To account for testing four variables, we performed a false discovery rate (FDR) correction using the initial p -values reported in the models that excluded interactions, as we only detected significant main effects. We used the function *p.adjust* and specifying a "fdr" method. We provide the results both before and after FDR correction.

2.5.2 | Offspring traits

We analysed data from 457 offspring from 128 different mothers' second broods. Linear models, LMM and GLMM, were run to determine the effect of male status and body size on: (a) offspring sex ratio

($n = 128$, binomial error with the *cbind* function [number of sons/daughters]); (b) offspring survival ($n = 457$, binomial error, 1 = alive / 0 = dead); (c) size at birth ($n = 446$, Gaussian); (d) growth rate ($n = 407$, Gaussian, power transformed). All models included initial maternal SL as a covariate, and mother identity as a random factor for models 2–4. Model 3 included brood size as covariate. We did not conduct a FDR correction as no fixed model terms were significant in any model.

2.5.3 | Son's reproductive performance

We analysed data from 196 sons from 109 mothers. We used LMM with Gaussian error distribution and maternal identity as a random factor to test for the effect of male status and body size on: (a) age at maturity ($n = 192$, log-transformed); (b) size at maturity ($n = 186$); (c) relative gonopodium length ($n = 185$, power transformed); (d) sperm number ($n = 174$, square-root transformed); (e) sperm velocity ($n = 178$, log-transformed).

Son attractiveness and mating behaviour were analysed using GLMM and LMM for: (a) the proportion of time females spent associating with the focal son in two-choice trials ($n = 168$, binomial error using the *cbind* function [time with son/time with competitor male]); (b) time spent harassing a female ($n = 181$, Gaussian error, square-root transformed); (c) number of gonopodial thrusts ($n = 181$, Poisson error); and (d) proportion of sons that performed a successful thrust ($n = 181$, binomial error, 1 = contacted female gonopore, 0 = no successful contacts). All models included maternal identity as a random factor.

To account for testing nine traits, we again performed a FDR correction using the initial p -values reported in the models that included interactions when these were significant, and the p -values of the main effects when interactions were nonsignificant. We provide the results both before and after FDR correction.

3 | RESULTS

Summary statistics and parameter estimates are provided in Table S1–S3.

3.1 | Female fecundity, growth and immune response

Females housed in the continual presence of a male were significantly less likely to give birth (Figure 3a) and had significantly lower fecundity (Figure 3b), slower growth (Figure 3c) and weaker immune responses (all $p < .015$) (Figure 3d) (Table S1). However, whether or not males were able to copulate (i.e. ablated or intact) did not affect any of these female traits (Tukey's tests, all $p > .62$). There was also no significant effect of male body size or any interaction between body size and male status (absent, ablated, intact) on the four measures of female fitness (all $p > .136$) (Table S1). All the variables remained significant after the FDR correction (all p -values $< .001$).

3.2 | Effects on offspring

3.2.1 | Offspring size at birth, early growth rate and the offspring sex ratio

Neither male status (absent, intact, ablated) nor male body size had a significant effect on offspring size at birth, growth rate or survival. There was also no interactive effect of male status and size on these measures of offspring performance, nor on the estimated birth sex ratio (Table S2). This suggests that neither an elevated mating rate nor the level of male harassment a female experienced affected the quality of her offspring. The lack of an effect of male size further suggests that there are no genetic benefits arising from offspring being sired by larger males, at least for the measured traits.

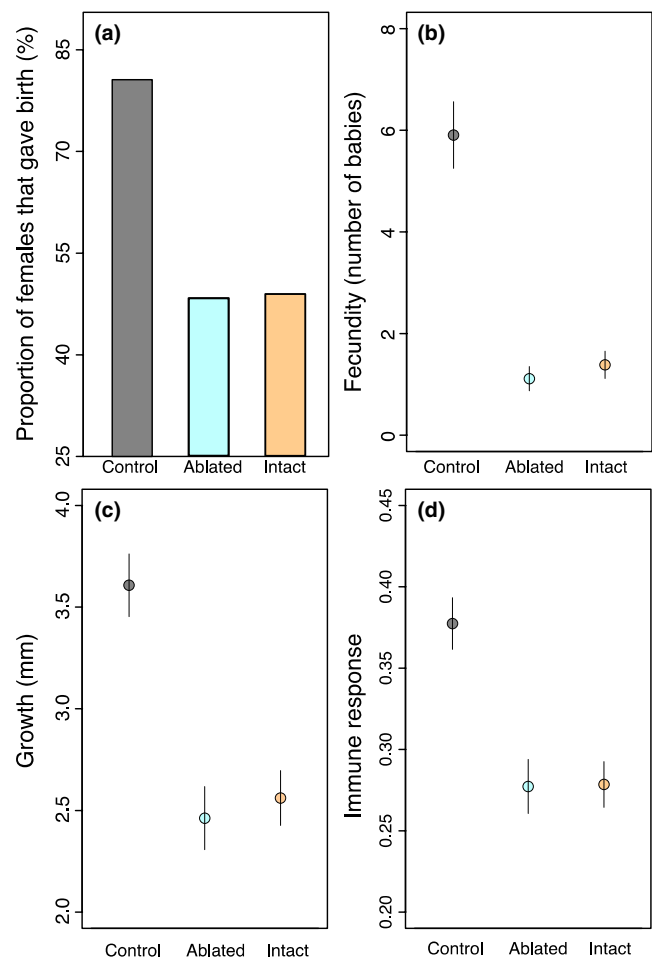


FIGURE 3 Effect of male status (absent: grey, ablated: blue, intact: orange) on female traits and performance: (a) proportion of females that gave birth ($n = 220$); (b) fecundity measured as the total number of babies born (excluding the first brood, $n = 134$); (c) growth (difference in female size between the last day of experiment and the size after the giving birth the first brood, $n = 274$); (d) immune response (PHA assay, $n = 275$). Data are presented as mean \pm SE

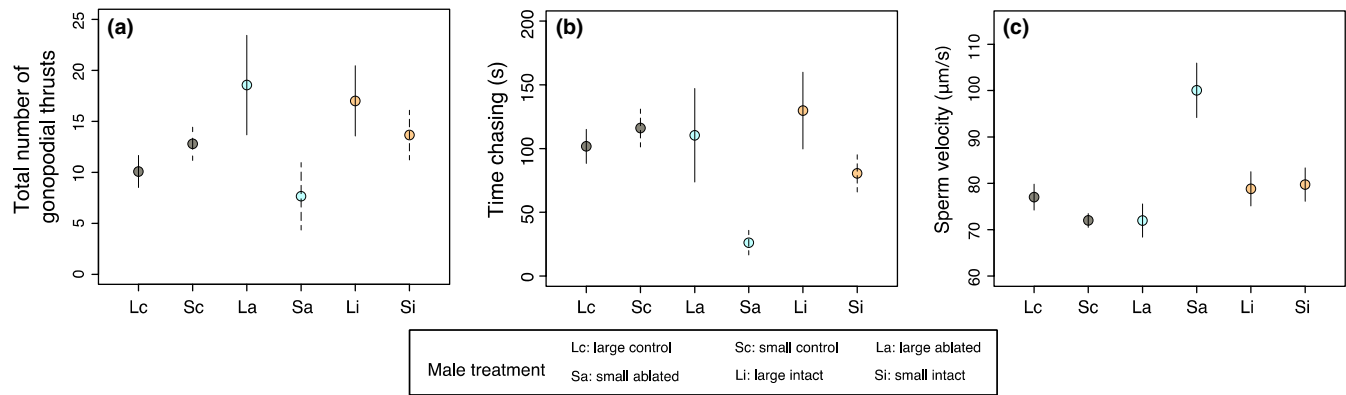


FIGURE 4 Effect of male size (large: solid line vs. small: dashed line) and status (absent: grey circles, ablated: blue circles, intact: orange circles) on sons' mating behaviour and sperm traits: (a) total number of gonopodial thrusts when housed with a female ($n = 181$), (b) time spent chasing females ($n = 181$), (c) sperm velocity ($n = 178$). Data are presented as mean \pm SE. The figures represent the raw data before the false discovery rate correction. After such correction, only the sperm velocity remained significant

3.2.2 | Son's performance

There were no significant effects of male size, male status or their interaction on relative gonopodium length, time to maturity, size at maturation, attractiveness, the proportion of sons that made successful gonopodial thrusts or sperm number (Table S3). There were, however, significant interactions between male status and body size that affected the number of gonopodial thrusts, the time spent chasing females and sperm velocity (Table S3). The sons of females housed with small, ablated males performed fewer gonopodial thrusts than those whose mothers were housed with small, intact males (Tukey's test, $p = .043$; Figure 4a), and they spent less time chasing females than the sons of mothers who were housed either with large, intact males (Tukey's test, p -value = $.013$; Figure 4b) or without a male (Tukey's tests, both p -values < $.033$). Finally, the sons of mothers housed with small, ablated males had faster swimming sperm than those of mothers inseminated with sperm from large males, regardless of whether they had been housed in the presence of intact (Tukey's test, p -value = $.041$) or ablated males (Tukey's test, p -value = $.018$) or in the absence of males (Tukey's test, p -value < $.001$; Figure 4c). Of these three traits, only sperm velocity remained significantly affected by the interaction between male status and size after the FDR correction ($p = .003$; number of gonopodial thrusts, $p = .105$; time spent chasing, $p = .125$).

4 | DISCUSSION

The costs of exposure to males for females are expected to depend on the level of male harassment and any effects of a resultant increase in mating rate. The strength of these effects might vary with male body size. To test these claims, we experimentally manipulated the ability of small and large male mosquitofish, *G. holbrooki*, to copulate by surgically removing the tip of their gonopodium to prevent copulation. We then housed females with a large or small male or alone, and measured their performance, their reproductive output

and the performance of their offspring. As predicted, females continuously housed with males paid a cost. They had significantly slower growth, a weaker immune response and lower fecundity than females housed alone. This effect is likely to be the result of continuous male harassment, but we cannot completely discard other potential effects of male presence such as competition for food resources. We did not, however, detect any difference between females exposed to large and small males, or between females housed with intact males, that could both harass and copulate with females, and ablated males that could only harass females. This suggests that the costs of copulation itself are low. Similarly, there was little evidence that male size, harassment or an increased mating rate had any effect on offspring performance, as might be predicted if males impose costs that alter maternal effects (e.g., reduced maternal investment into eggs).

We found a strong effect of male presence on direct measures of female fitness (i.e., reproductive output) as well as surrogate measures of costs, namely growth and immune function, that are likely to influence fitness in the less benign conditions that fish encounter in the wild. Our results are consistent with several previous studies that show that greater exposure to males has detrimental effects on female reproductive success (Lew *et al.*, 2006; Rönn *et al.*, 2006; Edvardsson, 2007, but see Smith & Sargent, 2006). Few studies have, however, teased apart the extent to which these costs are due to greater male harassment or an elevated mating rate, as the two usually covary. One notable exception is a study of the seed beetle, *Callosobruchus maculatus*, that found that the fitness costs to females of exposure to several intact males that could both harass and copulate with females were higher than those incurred by females who were exposed to several ablated males that could not copulate or to a single intact male (den Hollander & Gwynne, 2009). This suggests that there is a cumulative negative effect of harassment and elevated mating rate in seed beetles. In contrast, our findings suggest that male-imposed costs in mosquitofish are mainly due to continuous harassment because there was no increase in the fitness costs for females that were housed with intact males compared to those housed with ablated males. That harassment is costly

is unsurprising given the high rate at which males attempt to mate, so that females are either continually swimming away from or actively attacking males. These are both energetically costly activities that reduce the resources that can be allocated to reproduction and self-maintenance. The lack of a detectable effect of a greater mating rate is more surprising, as copulations in *G. holbrooki*, as in other poeciliid fishes, appear to damage the female oviduct (Sommer-Trembo *et al.*, 2017). Physical damage could plausibly create immune challenges due to secondary infections (Morrow & Innocenti, 2012).

One potential weakness of our study is that, although experimental fish were fed ad libitum, females housed with males might have experienced greater feeding competition than those housed alone (i.e., control females). When designing the experiment, we deliberately decided *not* to place another female in the control female tank as has been done in studies on guppies (i.e., presence of competitor, but no sexual interactions, e.g., Gasparini *et al.*, 2012) because previous studies suggest that social interactions between female mosquitofish can strongly affect their growth and fecundity (Smith, 2007; Fox *et al.*, 2019, Brookes, Iglesias-Carrasco, Kruuk, & Head, in review). One of our results also militates against the argument that male presence increases feeding competition: larger males are likely to be stronger competitors, but there was no moderating role for male size on the effect of male presence on the measured female traits.

Our finding that male size had no detectable effects on female fitness was unexpected. It has previously been shown that females prefer to associate with the larger of two males when given a choice (Kahn, Livingston, & Jennions, 2012; Kahn *et al.*, 2010) and one explanation is that large males are socially dominant and protect females from the continuous chasing and more frequent copulation attempts of smaller males. We therefore expected females housed with smaller males to incur greater costs if male harassment reduces their feeding rate and increases the energy devoted to avoiding and repelling males, but this was not the case. One open question is, however, whether our results would be the same if females were simultaneously housed with several males. In general, high male densities reduce female harassment due to increased direct male–male competition (Pilastro *et al.*, 2003; Smith, 2007). If male size affects the relative amount of time males spend defending versus attempting to mate with females, density-dependent behavioural variation might also vary with male size. Future studies could look at how our results translate into the natural setting and whether they can be linked to natural variation in the adult sex ratio to explain local variation in female fecundity and longevity.

The presence of males and their ability to mate did not have straightforward effects on any of the measured offspring traits. We expected that the strong detrimental effect of male presence on females (Figure 2) would, in turn, affect offspring quality. For example, continuous male harassment might elevate the production of stress hormones that, at least in other species, affect a range of offspring traits (Ensminger, Macleod, Langkilde, & Sheriff, 2018; Sheriff & Love, 2013). Similarly, differences in a female's future survival prospects when they are in poorer condition, independently of whether her poor condition is the result of harassment or food limitation, should

alter their current investment into offspring (Bowers, Bowden, Sakaluk, & Thompson, 2015; Brannelly, Webb, Skerratt, & Berger, 2016). Instead, despite the direct effects of exposure to males on females, there was no obvious decline in offspring quality. This contrasts with other studies that report detrimental cross-generational effects of male presence on offspring. For example, in seed beetles *C. maculatus* the daughters of harassed females had lower lifetime reproductive success than those mated once or multiply, although this effect was reversed in the following generation (Zajitschek *et al.*, 2018). Similarly, in guppies, *P. reticulata*, increased exposure to males lowered the reproductive success of their sons (Gasparini *et al.*, 2012). One factor that might explain the contrasting results between our study and that of Gasparini *et al.* (2012) (aside from species-specific effects) is that they measured offspring from a female's fourth rather than second brood. Their females had therefore spent longer in the corresponding treatments before producing offspring. These contrasting results highlight the potential importance of taking into account different trade-offs in female resource allocation (e.g., early vs. late reproduction, and immune response vs. reproduction), when exploring cross-generational effects.

Finally, there was little evidence that male size influences sons' mating behaviour or attractiveness. This is slightly unexpected as there is evidence of genetic heritability of male size in some poeciliids (Hughes, Rodd, & Reznick, 2005; Reznick, Shaw, Rodd, & Shaw, 1997). However, we have found no evidence for heritability of body size in our mosquitofish population (Vega-Trejo, Head, Jennions, & Kruuk, 2018). If body size is heritable, it should lead to differences in reproductive traits that are correlated with body size. For example, small males produce less sperm than larger males (O'Dea *et al.*, 2014). However, we found no effect of male size on sons' size at maturity (see also Vega-Trejo *et al.*, 2018; Zulian, Bisazza, & Marin, 1993), and the only significant findings related to male size after the FDR correction were the faster sperm velocity of sons whose mothers were exposed to small males that had been ablated compared to the sons of other types of mothers. However, there is no easy explanation for these results. Future studies might benefit from testing if the likely increase efforts of small ablated males to harass females might lead to the sons of such females doing better under sperm competition and gaining higher paternity. Additionally, it would be interesting to explore whether the effects on offspring traits vary with changes in the adult sex ratio. This is because in groups with similar male and female numbers, small males tend to be more successful at inseminating females, but larger males monopolize access to females in male-biased groups (Bisazza & Marin, 1995). Therefore, variation in the adult sex ratio might have important consequences for the evolution of local populations if heritable traits associated with male body size affect offspring phenotype.

5 | CONCLUSIONS

Our study substantiates past findings that greater exposure to males can lower female reproductive success. We showed that male

harassment, rather than physical damage directly associated with mating, imposes costs on females. The reproductive cost for females manifested as lower offspring production, but without a decline in offspring quality. Whether this result is generalizable across taxa, especially those where seminal fluids contain toxic chemicals or beneficial nuptial gifts, remains to be tested. It is worth noting that the allocation strategy of lower production of equivalent quality offspring that we observed might affect population growth in male-biased populations if fewer offspring are produced to maintain quality. Although we did not find any readily interpretable effects of greater exposure to males on offspring traits, the interactions that we did observe highlight the importance of investigating possible changes in maternal effects in response to variation in exposure to males of different phenotypes.

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CONFLICT OF INTEREST

We declare no conflict of interest.

AUTHOR CONTRIBUTIONS

MLH and MDJ conceived the study. MIC, RJF, MDJ and MLH designed the experiment. MIC, RJF and RVT collected the data. MIC and MLH analysed the data. MIC led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA AVAILABILITY STATEMENT

Data will be available through the Dryad Digital Repository (data-dryad.org). <https://doi.org/10.5061/dryad.46j4r0q>.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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