Original Article

Disentangling the costs of male harassment and the benefits of polyandry for females

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Many studies quantify how polyandry affects female fitness by allowing females to either mate with one or several males. But even if the number of matings is standardized, such studies conflate any costs of interacting with males with the potential benefits of receiving sperm from several males, obscuring the benefits of polyandry. We conducted a 2×2 factorial experiment on the mosquitofish, *Gambusia holbrooki* to quantify the independent effects of male harassment and polyandry. We artificially inseminated virgin females with sperm from either 1 or 5 males (monandry vs. polyandry). Females were then housed in the presence or absence of reproductively ablated males who could harass, but not mate with them. Our design ensured that the number of males inseminating a female was independent of the level of male harassment and the number of matings she received. Females who were not housed with males were instead housed with immature females to maintain densities constant across treatments. Unexpectedly, females that experienced sexual harassment were more likely to give birth, had shorter gestation periods and gave birth to larger broods. Furthermore, polyandrous females were more likely than monandrous females to give birth. Polyandrous females' sons also reached maturity faster than those of monandrous females. We therefore found no detectable costs to females producing their first brood of being harassed by males when the direct costs of mating were absent. We also showed that, in the absence of mating costs, there are direct and indirect fitness benefits of being inseminated by multiple males. If the costs of the act of mating are small or absent, polyandry will benefit female *G. holbrooki* producing their first brood.

Key words: **coercive mating, convenience polyandry, *Gambusia holbrooki*, maternal effects, Poeciliidae, sexual conflict.**

INTRODUCTION

In many species, females can fertilize all their eggs using sperm from a single mating. This begs the question of why females are polyandrous and mate with several males (Jennions and Petrie 2000; Zeh and Zeh 2003; Simmons 2005; Pizzari and Wedell 2013). There are 2 main explanations for polyandry. First, there are genetic benefits if paternity is biased towards males who sire offspring of above average fitness. This could occur if cryptic female choice favors fertilization by genetically compatible sperm (e.g., Løvlie et al. 2013; review: Firman et al. 2017); or if sperm competition selects for males that produce competitive ejaculates, and this trait is correlated with net fitness (e.g., Evans et al. 2003; Hosken et al. 2008; Pizzari and Parker 2009, but see Danielsson 2001). Second, there is usually an imbalance between the optimal mating rate of males and females because males increase

their reproductive success with each successive mating, while there are diminishing benefits of each additional mating for females (Clutton-Brock and Parker 1995; Chapman et al. 2003; Arnqvist and Rowe 2005; Parker 2006; Janicke et al. 2016). This often manifests itself in sexual conflict over specific mating decisions, leading to male sexual harassment and coercive mating. Females may therefore mate multiply, either because they reduce any immediate costs to their fecundity or lifespan by acquiescing to persistent male harassment ("convenience polyandry" sensu Thornhill and Alcock 1983; Arnqvist and Rowe 2005), or simply because males engage in forced copulation (Arnqvist 1989; Le Boeuf and Mesnick 1990; Morrow and Arnqvist 2003). To distinguish between explanations for polyandry based on benefits versus avoidance of costs, we need to know the relative magnitude of the costs of sexual harassment.

To date, most studies have conflated the effects of sexual harassment with the benefits of polyandry when trying to quantify the effect on female fitness of being able to mate with multiple males. Many early studies were correlational and asked if multiple paternity predicted offspring performance (e.g., Madsen et al. 1992; Rodriguez-Muñoz et al. 2010), while other studies simply housed

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females with either one or several males and then compared their offsprings' performance (e.g., Gowaty et al. 2010). In both types of studies, the number of actual and/or attempted matings was conflated with whether or not a female was polyandrous. Later studies controlled for the number of matings per female either experimentally, by comparing females mated N times with one male to females mated with N males once each (meta-analysis: Slatyer et al. 2012), or by taking advantage of mating systems with external fertilization and satellite males that release sperm without mating (e.g., Byrne and Whiting 2011). In a few cases, artificial insemination was used to control for any direct effects of male presence, such as females adjusting their reproductive allocation in response to how many males they encountered (Sheldon 2000; Simmons 2005; Seeley and Tarpy 2007). In general, these studies showed that polyandrous females have higher reproductive output and offspring viability than monandrous females (e.g., Fisher et al. 2006; Eizaguirre et al. 2007; Gowaty et al. 2010). Even when confining the analysis to studies that fully control for the number of matings per female, there is still a small, significant benefit to polyandry (meta-analyses: South and Lewis 2011; Slatyer et al. 2012; Taylor et al. 2014).

To demonstrate that females mate multiply to reduce the costs of resisting male mating attempts (i.e., "convenience polyandry"), it is necessary to show a cost to females of male sexual harassment and prolonged mating resistance (Huchard et al. 2012). But what is the evidence that harassment is costly? Initial studies highlighted that males can directly physically damage females (e.g., oviduct damage from male genitalia in seed beetles, Crudgington and Siva-Jothy 2000, or reduce receptivity due to seminal toxins in *Drosophila*, reviewed by Chapman 2001), or that avoiding male harassment can reduce the time spent foraging or evading predators (e.g., Pilastro et al. 2003; Plath et al. 2007; Galimberti et al. 2000). Later studies showed that male-induced damage can lower female reproductive output (e.g., Le Galliard et al. 2008; Sakurai and Kasuya 2008; Takahashi and Watanabe 2010; Gay et al. 2009; Rossi et al. 2010) or longevity (Le Boeuf and Mesnick 1990). The evolution in females of morphological traits or "male avoidance" behaviors that seemingly protect them from male aggression (e.g., defensive "shields" in bedbugs (Morrow and Arnqvist 2003); solicitation of protection from mates or dominant males (Mesnick and Le Boeuf 1991; Clutton-Brock and Parker 1995; Dadda et al. 2005, 2008); and altered foraging patterns (Pilastro et al. 2003; Wearmouth et al. 2012) are all suggestive that sexual harassment is costly. Even so, male and female behaviors indicative of "sexual conflict" are not always costly to females (e.g., Head and Brooks 2006; Galimberti et al. 2000; Gasparini et al. 2012; Helinski and Harrington 2012). For example, "excess" mating had no significant effect on the reproductive rate of females in 3 insect species (Morrow et al. 2003), and females showed no decrease in foraging rate while being harassed by males in topminnows (Dadda and Bisazza 2006). A few studies have compared the fitness of females housed with one or N males, or housed with males for varying periods of time, and explicitly described this in terms of "level of male harassment" (e.g., Gasparini et al. 2012). The problem, however, is that females subject to higher levels of harassment are also likely to mate more often, and with more males: polyandry and harassment are therefore conflated. If polyandry confers benefits, this could lead to the costs of harassment and/or the benefits of polyandry being underestimated if harassment costs are nonadditive when imposed by multiple males (Carazo et al. 2014).

Ideally, sexual harassment and polyandry should be independently manipulated to determine their respective effects.

Unfortunately, many studies allow harassment level, number of mates and mating rate to covary (e.g., Edvardsson 2007; Le Galliard et al. 2008; Makowicz and Schlupp 2013). But male harassment and polyandry can be experimentally disentangled using ablated males to calculate the effect of harassment/excess mating, and artificial insemination can be used to control for the number of males inseminating females. If these ablated males are additionally incapable of mating, we can identify the costs of sexual harassment beyond those associated with the mating act. To our knowledge, only one study has simultaneously examined the costs/benefits of male sexual harassment and polyandry (i.e., number of males inseminating) while controlling for the mating rate (Zajitschek et al. 2018).

Here, we investigate the effect of male harassment and polyandry on female eastern mosquitofish (*Gambusia holbrooki*) (Poeciliidae). Males rarely court, and mating is coercive (Bisazza 1993; Plath et al. 2007). Males chase females and attempt to forcibly inseminate them using their modified anal fin ("gonopodium"), whose distal end is characterized by spines that probably assist in sperm transfer (Kwan et al. 2013). These spines can damage the female's genitalia (Sommer-Trembo et al. 2017; R.J.F., personal observation). Males persistently attempt to mate, at rates of up to one attempt/minute (Wilson 2005). Although females are polyandrous (Booksmythe et al. 2016; Head et al. 2017) they mainly try to evade insemination, and often attack males that are attempting to copulate (Pilastro et al. 2003). Females store sperm derived from several males (Constantz 1984) and the potential for cryptic female choice is therefore increased (e.g., as in guppies, Pilastro et al. 2004). The mating system is characterized by persistent male harassment *and* postcopulatory sexual selection, meaning that polyandry and harassment are usually inextricably linked. By breaking the link between the 2 under controlled laboratory conditions, we are therefore able to test how polyandry affects female fitness *independent of* male sexual harassment. Our aim was to establish the relative effects of male harassment and polyandry on female *G. holbrooki* producing their first brood, including any effects on their offspring's performance. To ensure that the effects of sexual harassment were not conflated with those of multiple mating, we either housed focal females with males (i.e., male harassment present), who were experimentally modified so that they could neither inseminate nor mate, or with male-sized immature females (i.e., male harassment absent). We then used artificial insemination of these virgin females to manipulate the actual level of polyandry. We considered only the first brood produced by females, since further rounds of insemination, combined with the ability of female *G. holbrooki* to store sperm, would have hampered our ability to explicitly control the level of polyandry (requiring either a 6-month gap between inseminations, or that the exact same males be used to reinseminate individual females, which was not feasible without a large reduction in sample size due to the shorter lifespan of males). We predicted that: 1) male harassment would lower female reproductive output and reduce offspring fitness as has been reported in other species (e.g., guppies, Gasparini et al. 2012; seed beetles, Zajitschek et al. 2018); 2) polyandrous females would have higher reproductive output and offspring fitness than monandrous females; and 3) that the beneficial effects of polyandry would be greater for nonharassed than harassed females due to differential effects of harassment on females across mating levels cf. differential effects on female longevity of harassment across mating levels (Wilson and Tomkins 2015).

METHODS

To generate our breeding stock, we collected pregnant eastern mosquitofish, *G. holbrooki* from Sullivan's Creek, Canberra, Australia and transferred them to our aquarium facilities at the Australian National University. The females were placed in individual 1-L tanks containing a mesh divider to provide a refuge for offspring. Tanks were checked twice daily for newborn fry, which were then transferred to 90-L aquaria (up to 50 individuals per aquarium). These offspring were reared until we could sex them, after which they were segregated into single-sex tanks prior to being used in experiments to ensure that all females were virgins. All stock and experimental fish were housed at 28 °C (±1 °C) under a 14 h light:10 h dark cycle, and fed commercial fish flake (morning) and brine shrimp (afternoon) ad libitum (stock fish), or brine shrimp ad libitum twice daily (experimental fish).

Experimental setup and protocols

Our experiment had a 2×2 factorial design such that each focal virgin female either did or did not experience continual sexual harassment from ablated males who could neither inseminate nor mate, and she was artificially inseminated by sperm from either a single male (monogamy) or 5 males (polyandry) ($n = 4 \times 40 = 160$ females). We could therefore test for independent effects of male harassment and polyandry on female reproduction, and treatment-dependent maternal effects on offspring traits.

In total, 160 adult virgin females from the laboratory-raised stock were weighed (±0.1 mg), measured (standard length ±0.01 mm), and then randomly assigned to treatment groups. There was no difference between treatments in either the initial mass or standard length of females ($F_{3,156} = 0.024$, $P = 0.995$; $F_{3,156} = 0.034$, $P = 0.992$). Females in the sexual harassment treatment were housed in 7.5-L aquaria with 3 ablated males who were unable to transfer sperm, but otherwise exhibit the same level of harassment towards females as intact males (Mautz 2011). The process

of ablation was carried out by anesthetizing males in an ice-water slurry and removing the distal tip of their modified anal fin ("gonopodium") using a sterile scalpel blade. Males were then placed in individual 1-L tanks with aeration to recover from anesthesia, before being transferred back to single-sex stock tanks for a recovery period. The procedure was carried out 5 days prior to the start of the experiment, although in practice males exhibit normal behavior almost immediately, that is, attempted to copulate with available females within minutes of recovery from anesthesia (observed by ANU Veterinarian). The procedure was conducted under approval from the ANU Animal Ethics Committee and had a 100% survival rate. Following commencement of the experiment, males were exchanged at random between harassment treatment tanks every 7 days to provide them with the stimulus of a novel potential mate and thereby maintain high harassment levels of females over the 28-day treatment. In the no sexual harassment treatment, focal females were housed in 7.5-L aquaria with 3, immature females of a similar size to the males used in the harassment treatment (mean SL = 23.6 ± 0.2 mm, compared to mature males mean SL 24.1 ± 0.3 mm), and also transferred between tanks every 7 days. The inclusion of immature females minimized any difference between the treatments in fish biomass and competition for food (see Gasparini et al. 2012 and references therein). Mosquitofish are social, so the presence of immature females also alleviated any stress that focal females might otherwise have experienced if housed alone (Evans et al. 2007). It should be acknowledged, however, that females in the no sexual harassment treatment may have experienced intrasex competition as an unavoidable side-effect of countering the issues outlined above. We specifically used immature females to minimize the effects of intrasex competition.

After 14 days, the 80 females from each of the 2 harassment treatments were divided into 2 groups and artificially inseminated with sperm from either 1 (monandry) or 5 males (polyandry), yielding 4 treatment groups of 40 individuals each (harassed/monandrous, harassed/polyandrous, nonharassed/monandrous
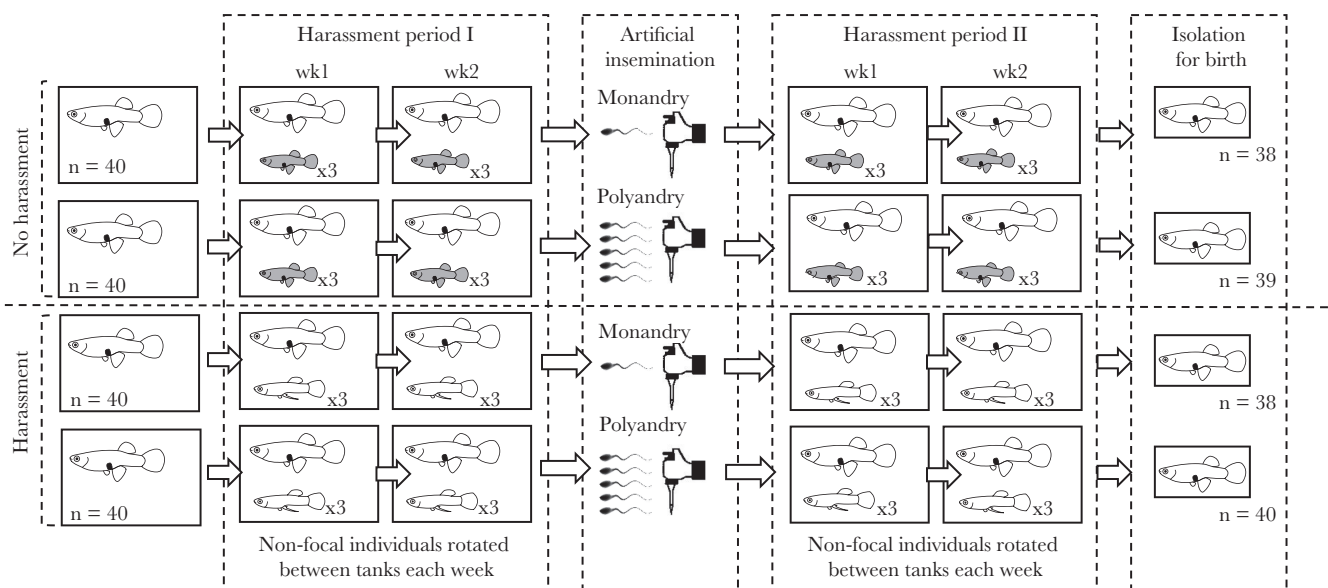

Figure 1
Experimental protocol to determine the effects of male sexual harassment (by an ablated male) and polyandry on reproduction of female eastern mosquitofish (*G. holbrooki*). Females are represented by fish with a black gravid spot. Immature (nonfocal) females are represented in grey. Males are represented by fish with an extended anal fin (the gonopodium) that had been ablated.

and nonharassed/polyandrous) (Figure 1). This method removed any confounding costs arising from male harassment and/or the act of mating by controlling for both the mating rate and cost of mating for each female (all females experienced a single insemination/mating event). It also ensured that females in the polyandry treatment were inseminated by multiple males (as opposed to making the assumption that this will automatically occur if a female is housed with multiple males, or having to argue that contact between male gonopodium and female gonopore equates to a successful transfer of sperm, which is not always true for *G. holbrooki* (R.J.F., personal observation). The sperm used were taken from males from our general stock. The level of polyandry (5 males) we used was higher than current estimates of the number of males siring a brood in wild populations of *G. holbrooki* (1–3 sires/brood). This was done to account for the fact that the number of sires in paternity studies tends to underestimate the number of mates (cf. Zane et al. 1999; Dean et al. 2006). To artificially inseminate females, we first anesthetized each male in ice water and placed him on a PVA-coated glass slide under a dissection microscope. We then swung the gonopodium forward until it was perpendicular to his body. Sperm was extracted by applying gentle pressure to the abdomen to release ejaculate onto the slide, and 100 μL of 0.9% NaCl solution was then added to keep the ejaculate hydrated. An exact number of sperm bundles (5 or 25) were collected from each male's ejaculate using a micropipette and temporally placed in a microtube containing 2 μL of saline solution (minimal volumes of seminal fluid were transferred with the sperm bundles). For the monandry treatment, 25 sperm bundles were collected from a single male and inseminated into a focal female. Focal females assigned to the polyandry treatment were inseminated with the same volume of solution containing 25 sperm bundles extracted from 5 males (5 bundles per male). To inseminate females, we allowed sperm bundles to settle together at the bottom of the microtube and then used a 3-μL micropipette to draw up all of the solution and insert it into the reproductive tract of an anethetized female. Although *G. holbrooki* are generally considered to be lecithotrophic, exposure to male harassment in both the pre- and postinsemination period is required to ensure that we estimated any indirect effects on maternal changes in the fluid surrounding the eggs (e.g., stress hormone levels) (Wourms 1981). Focal females were therefore returned to their individual tanks for another 14 days of their respective harassment treatment (again with weekly exchange of males between tanks, Figure 1).

After the 28-day treatment period, we anesthetized nonfocal males and photographed their gonopodium to confirm the absence of distal spines at the tip. None were present, which confirms that the test males had not inseminated focal females (Mautz 2011). Focal females were transferred to individual 1-L aquaria containing a mesh divider to create a refuge for any newborn offspring. Over the next 50 days, tanks were checked twice daily for offspring. Any fry were immediately removed, photographed individually from above in a petri dish placed over 1 mm graph paper, and then transferred to individual 1-L tanks. At 28 days of age, each juvenile was rephotographed. Every 3 days for 5 months, all offspring were inspected to determine when they reached sexual maturity. For males, this was defined by the presence of a fully-formed gonopodium (including distal spines); and for females by the presence of a visible gravid spot on the ventral flank. Sexually mature individuals were rephotographed to measure their size at maturity.

Measuring female reproductive output and offspring development

We recorded the effects of male harassment and polyandry on 2 measures of female reproductive output: gestation period and fecundity. Gestation period was the number of days between insemination and when fry were born, while fecundity was the number of fry produced (brood size). To examine potential intergenerational effects of sexual harassment by males and polyandry, we recorded 4 offspring traits for all available offspring: 1) size at birth (standard length), 2) growth rate (the increase in standard length by day 28), 3) time to maturity, and 4) size at maturity (standard length). We also noted the sex of the offspring.

Statistical analyses

Five females were excluded from our analyses (2 died and 3 had unsuccessful artificial inseminations). Final sample sizes were therefore: harassed/polyandry ($n = 40$); harassed/monandry ($n = 38$); nonharassed/polyandry ($n = 39$); and nonharassed/monandry ($n = 38$) (Figure 1). To determine how female reproductive output was influenced by male harassment and polyandry, we ran 3 separate analyses. First, we tested for the effects of male harassment and polyandry on whether or not a female gave birth using a generalized linear model with binomial error structure. Then, using data from females that gave birth ($n = 49$ from male harassment treatment, $n = 11$ from no male harassment treatment), we tested the effects of harassment and polyandry on gestation period using a general linear model (Gaussian error structure), and on brood size using a generalized linear model (Poisson error structure and log link function). In all cases, harassment and polyandry treatments were treated as fixed factors and female body mass was a covariate. We included the 2-way interaction between harassment and polyandry in the initial model, but if it was nonsignificant, we interpreted the main effects of a reduced model excluding the interaction term.

Nonharassed females produced only 19 of 247 offspring (see Results), which prevented us from examining the effect of male harassment on offspring traits. We therefore only examined the effect of polyandry on offspring traits for mothers who had been harassed. To determine the effect of polyandry on offspring 1) size at birth, 2) growth rate, and 3) size at maturity, we ran separate linear mixed effects models (Gaussian error structure), with polyandry and offspring sex as fixed effects, and maternal identity as a random factor because we measured multiple offspring from the same brood. Brood size was included in all the models as a covariate. Brood size could, however, be considered an intermediate variable (i.e., directly affected by the treatments); so, we reran all models excluding brood size to confirm the main treatment effects. The results were equivalent; so, we present models including brood size. To test the effect of polyandry on 4) offspring time to maturity (in days), we ran a generalized linear mixed effects model with Poisson error structure. In all 4 cases, we included the interaction between polyandry and offspring sex in the initial model, but if it was nonsignificant, we interpreted the main effects from a reduced model excluding the interaction term. Model residuals were examined to confirm they approximated a normal distribution when assuming a Gaussian error distribution. Models assuming a Poisson error structure were checked to confirm they were not overdispersed. All analyses were conducted in R version 3.4.0, using the *lme4* package to run linear mixed effects models and the *lmerTest* package to obtain *P*-values and confidence intervals.

RESULTS

Effects of male harassment and polyandry on breeding

Of the 155 females that were artificially inseminated, 60 produced broods, for a total of 247 offspring. Unexpectedly, females that experienced male harassment were significantly more likely than those who did not experience sexual harassment to produce offspring (Table 1) (49 of the 60 broods produced were from harassed females). Likewise, polyandrous females were significantly more likely than monandrous females to produce offspring (36 of the 60 broods produced were from polyandrous females) (Table 1, Figure 2). There was no significant interaction between the occurrence of male harassment and polyandry on whether or not a female bred (Binomial GLM, $Z = 0.521$, $P = 0.602$); nor was there a significant effect of her initial mass (Table 1).

Females that experienced male harassment had significantly shorter gestation periods and produced significantly larger broods than nonharassed females (Figure 3a, Table 1). These effects were not attributable to differences in either size or mass ($t_{58} = -0.319$, $P = 0.375$; $t_{58} = -0.175$, $P = 0.431$) between harassed and nonharassed females that gave birth. In contrast to the effects of sexual harassment, there was no significant effect of polyandry on either gestation time or brood size (Figure 3b) and there was no interaction between the effects of male harassment and polyandry for either gestation time ($t = 0.211$, $P = 0.833$) or brood size ($Z = 1.348$, $P = 0.178$). Finally, larger females had significantly shorter gestation periods, but female size had no effect on brood size (Table 1).

Effect of polyandry on offspring development

Full details of the statistical analyses of factors that might affect offspring traits of harassed females are given in Table 2. Sons were significantly larger than daughters at birth (Figure 4), and maternal effects (i.e., maternal identity) explained a significant amount of the variation in offspring size at birth (Log-likelihood ratio test, $\chi^2_{(1)} = 63.95$, $P < 0.001$). There was, however, no effect of polyandry on either size at birth or growth rate; nor was there a significant interaction between polyandry and offspring sex for either size at birth ($t = 1.73$, $P = 0.086$) or growth rate ($t = 0.16$, $P = 0.873$). There was also no significant effect of brood size on either offspring size at birth or growth rate. Maternal effects (i.e., random effect of mother ID) were not significant in explaining variation in offspring growth rates (Log-likelihood ratio test, $\chi^2_1 = 2.51$, $P = 0.113$)

Polyandry and offspring sex had an interactive effect on both the size at which offspring matured, and the time taken to reach maturity. The daughters of polyandrous and monandrous females took a similar time to mature, but the sons of polyandrous females matured significantly faster, and at a significantly smaller size, than those of monandrous females (Figure 5). Finally, there was no significant difference in the offspring sex ratios produced by polyandrous and monandrous females (56.5% vs. 52.9% daughters; $z = 0.540$, $P = 0.589$), nor a biased overall sex ratio (54.8% daughters; $\chi^2_1 = 1.007$, $P = 0.316$).

DISCUSSION

We disentangled the effects of polyandry and sexual harassment on female fitness with an experimental design that explicitly examined the effect of male harassment after controlling for the number of mates, and vice versa, and also eliminated any costs of actually mating. There was no detectable cost to being harassed by males for female *G. holbrooki* producing their first brood. However, we found direct and indirect fitness benefits of being inseminated by multiple males. Our findings suggest that polyandry is, at least initially, beneficial for females. In nature, however, females constantly evade males, which suggest that there are costs associated with being inseminated by several males that arise from the act of mating, or that there are longer-term costs of male harassment that accumulate. Since female *G. holbrooki* can store sperm for up to 6 months, we only examined fitness effects of sexual harassment and polyandry associated with the production of their first brood. Although our results might underestimate long-term costs of male harassment that only become evident in subsequent broods (e.g., Gasparini et al. 2012), they have the advantage of providing a clear quantification of the benefits of polyandry unobscured by any costs of male sexual harassment or mating, which has been missing in other studies.

Effects of male harassment and polyandry on reproduction

Contrary to our predictions, we found a significant positive effect of male harassment on the reproductive output of females. Harassed females were more likely to give birth to a brood, and had shorter gestation periods and larger clutches than those who did not experience male harassment. Although surprising, this result agrees with the findings in guppies (*Poecilia reticulate*), where females had greater fecundity in early broods when they experienced higher levels of

Table 1

Parameter estimates for the effects of male harassment (yes/no), mating system (monandry vs. polyandry), and female body mass (covariate) on the reproductive output of female eastern mosquitofish (*G. holbrooki*): 1) whether or not a female produces offspring, 2) the gestation period, and 3) brood size

	Female gives birth			Gestation[a]			Brood size		
	Estimate	Std. Error	*P*	Estimate	Std. Error	*P*	Estimate	Std. Error	*P*
(Intercept)	0.125	0.306	0.683	34.202	1.481	**<0.001***	1.598	0.097	**<0.001***
Harassment (Non)	−2.428	0.420	**<0.001***	5.626	2.369	**0.021***	−0.967	0.240	**<0.001***
Mating syst (Poly)	0.812	0.399	**0.042***	−1.612	1.898	0.399	−0.111	0.130	0.392
Initial mass (mg)	−0.820	0.433	0.058	−4.583	1.862	**0.017***	−0.182	0.130	0.162

In all cases, there was no significant interaction between harassment level and mating system (see text) and significance values are from a reduced model without the interaction.
[a]A shorter gestation period is considered beneficial since it allows the female to produce more clutches over the reproductive season.

sexual harassment (Gasparini et al. 2012). In the same study, however, Gasparini et al. (2012) found that females who had experienced less male harassment produced larger later broods, whereas the brood size of females who experienced greater sexual harassment either remained stable or declined through time. This finding suggests that sometimes the costs of male harassment might only be revealed in the longer-term.

One explanation for the low rates of reproduction of females in the "no sexual harassment" treatment is that exposure to males is needed to "prime" females to breed. However, Marsh et al. (2017) also raised and artificially inseminated female *G. holbrooki* who had never been in the presence of a male and observed rates of reproduction of 56%: virtually indistinguishable from the 61% birth rate for females who had been exposed to males in the current study. Therefore, although there are currently no studies testing whether priming affects reproduction in mosquitofish, we suggest that this factor alone is unlikely to explain the low birth rate of nonharassed females in our study.
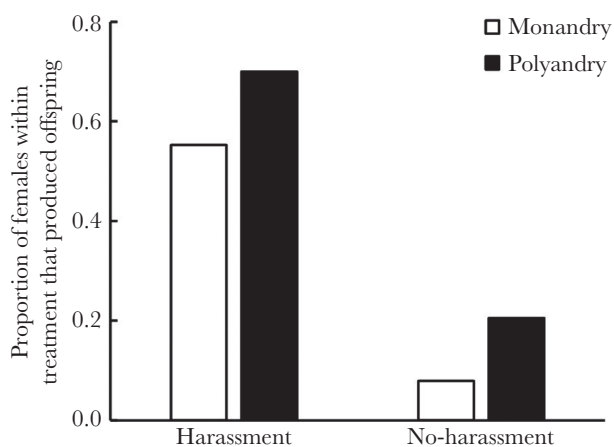

Figure 2

Effect of male harassment and polyandry on the proportion of females that gave birth in eastern mosquitofish (*G. holbrooki*) (N = 38 harassment/monandry, N = 40 harassment/polyandry, N = 38 no-harassment/monandry, and N = 39 no-harassment/polyandry).

Figure 3

Effect of male harassment and polyandry on: (a) the gestation period (days); (b) brood size of female eastern mosquitofish (*G. holbrooki*) within each treatment type that gave birth. Mean ± SE (N = 21 harassment/monandry, N = 28 harassment/polyandry, N = 3 no-harassment/monandry, and N = 8 no-harassment/polyandry).

Table 2

Parameter estimates for the effect of polyandry, offspring sex, and brood size on traits of offspring produced by female eastern mosquitofish (*G. holbrooki*) experiencing male harassment: 1) offspring size at birth (standard length in mm); 2) offspring growth rate over the first 28 days; 3) time to maturity (days); and 4) size at maturity (standard length in mm)

	Birth size			Growth			Time to mature			Size at maturity		
	Est.	Std. Error	*P*	Est.	Std. Error	*P*	Est.	Std. Error	*P*	Est.	Std. Error	*P*
(Intercept)	6.646	0.116	**0.000**	0.355	0.006	**0.000**	4.324	0.035	**0.000**	22.22	0.241	**0.000**
Polyandry (Poly)	0.176	0.149	0.232	0.005	0.007	0.469	0.065	0.046	0.158	0.480	0.317	0.134
Sex (Male)	0.196	0.071	0.006	−0.008	0.007	0.264	−0.020	0.028	0.474	−0.449	0.331	0.177
Brood size	−0.240	0.132	0.062	−0.001	0.006	0.835	−0.042	0.023	0.070	−0.018	0.238	0.939
Polyandry*sex							−0.222	0.037	**0.000**	−1.148	0.445	**0.011**

If there was no significant interaction between mating system and offspring sex (see text for statistics), significance values are from the reduced model run without this interaction. Maternal identity was included as a random effect to account for the fact that multiple offspring per mother were measured (see text). Values in bold are significant at the 0.05 level.
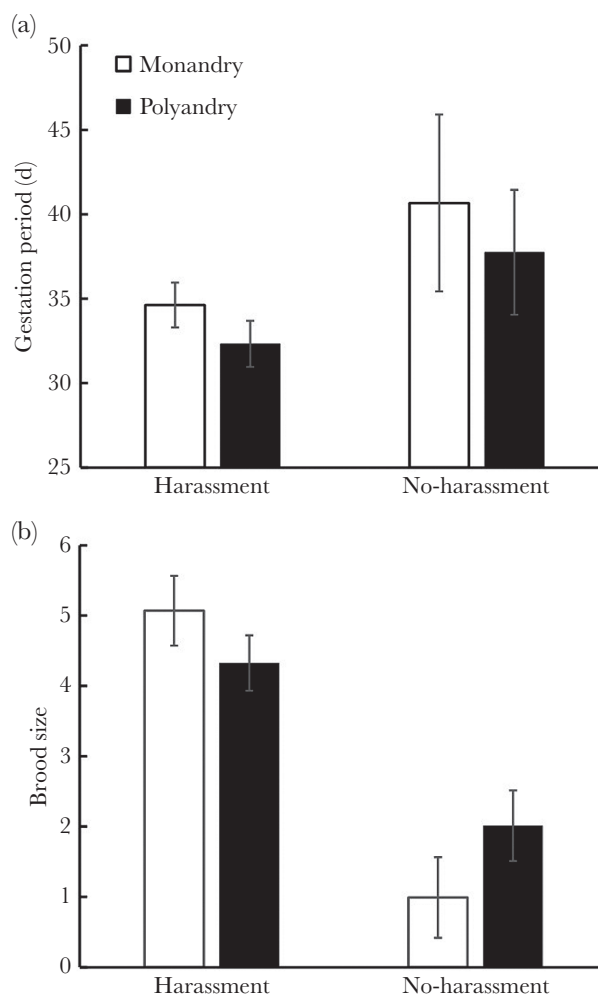
It is also important to note that we housed nonharassed females with juvenile females to minimize any treatment differences in competition for food or social isolation (cf. Evans et al. 2007). Our finding that being with females had a more negative effect on reproductive output than male harassment is consistent with findings from other poeciliids. For example, female growth and reproduction in *Gambusia affinis* decreased as the proportion of females in the population increased, and high female densities had a more detrimental effect on female fitness than male harassment (Smith 2007; Smith and Sargent 2006). In guppies, being housed with females incurred a higher reproductive cost than being housed alone, due to slower growth, slower egg maturation and fewer mature eggs (Borg et al. 2006, 2012). Nonetheless, our study suggests that intrasex competition and contests for dominance among females have a *more* negative effect than male sexual harassment on a female's short-term reproductive output (see also Head and Brooks 2006).

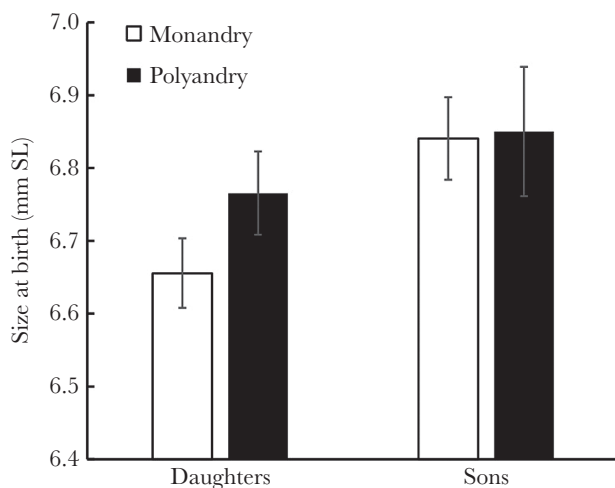For females that bred, polyandry did not significantly shorten the gestation period or increase brood size. This is counter to the general trend across taxa. For example, polyandrous guppies have shorter gestation periods and larger broods (Evans and Magurran 2000), and polyandrous females typically have larger clutches than monandrous females in lizards (Eizaguirre et al. 2007) and mice (Firman and Simmons 2008). Even so, in *G. holbrooki*, the net effect of polyandry on female reproduction was positive because females inseminated by multiple males rather than a single male were significantly more likely to breed. Similar findings in other species where males only transfer ejaculates to females (i.e., where mating does not affect parental care or access to nonejaculate linked resources) have been attributed to polyandry lowering the risk of receiving only infertile sperm (review: Hasson and Stone 2009); or to beneficial resources in ejaculates (South and Lewis 2011; Crean et al. 2016).

The observed net positive effect of being inseminated by several males raises the question of why female *G. holbrooki* usually evade male mating attempts in the wild. This behavior could be a form of mate choice to avoid less active males. It could also indicate that some costs of polyandry are directly due to the act of copulation (any such cost of mating was excluded from the current study). There are 2 obvious reasons why copulation could be costly. First, the distal spines of the gonopodium damage females, who sometimes bleed from their gonopore after being inseminated (Horth 2003; R.J.F., personal observation). Second, mating carries the risk of transmission of sexual infections (Kokko et al. 2002; Ashby and Gupta 2013). In general, one expects a species with high contact between the sexes to have many sexually transmitted diseases. Multiple mating is therefore likely to increase the risk of disease transfer (Ashby and Gupta 2013). Since both factors could reduce female fitness, the benefits of polyandry in our study are probably an overestimate, whose magnitude depends on the shape of the function relating the number of matings to the costs imposed on females (e.g., linear vs. exponential). Future studies will need to determine the nature of these relationships. Finally, in our study, any costs of resisting male sexual harassment were clearly lower than those imposed by female–female competition and maintaining social dominance (see above). If this is true in the wild, it might explain why females can afford to invest in resisting male mating attempts

Effect of polyandry on offspring fitness

Too few nonharassed females gave birth to test if male harassment and polyandry interact to affect offspring fitness. For harassed

Figure 4

Effect of polyandry on the size at birth (standard length in mm) of daughters and sons of female eastern mosquitofish (*G. holbrooki*) subjected to male harassment. Mean ± SE ($N = 55$ monandry daughters, $N = 65$ polyandry daughters, $N = 47$ monandry sons, $N = 50$ polyandry sons).
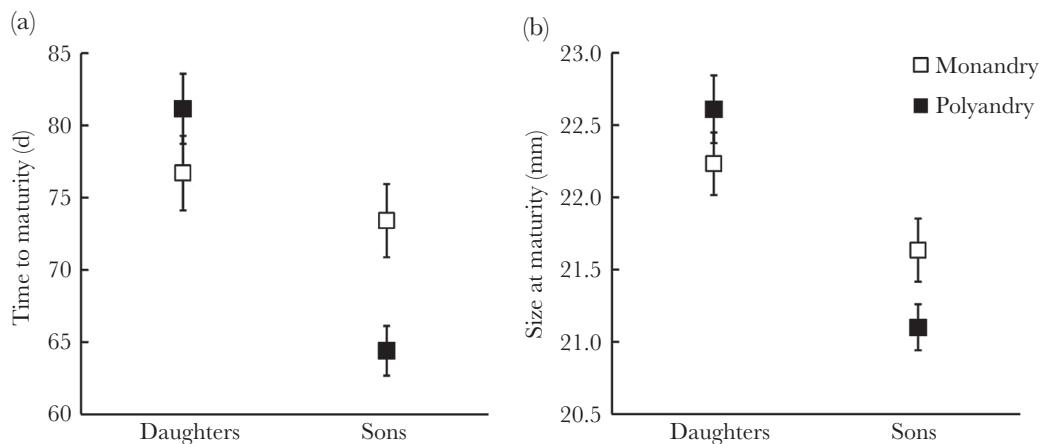

Figure 5

Effect of polyandry on (a) time to maturity (days) and (b) size at maturity (standard length in mm) of daughters and sons of female *G. holbrooki* that were harassed by males. Mean ± SE ($N = 55$ monandry daughters, $N = 65$ polyandry daughters, $N = 47$ monandry sons, $N = 50$ polyandry sons).

females, there was, however, an effect of polyandry on sons' performance. Polyandrous females' sons matured significantly faster, albeit at a smaller size, than those of monandrous females. This result is potentially attributable to sperm competition and/or cryptic female choice of sperm from smaller males, who tend to be more successful when competing in a natural setting (see below). Alternatively, it could be due to cryptic female choice for sperm that maximize genetic compatibility or heterozygosity. Future studies that control for the number of sperm inseminated through artificial insemination could directly test for a small-male fertilization advantage (although Locatello et al. (2008) did not find body size-related differences in ejaculate quality in another population of *G. holbrooki*). Alternatively, females might use the number of males contributing sperm to their reproductive tract as a cue to manipulate the developmental trajectory of sons. This type of maternal effect occurs in some species (e.g., Cunningham and Russell 2001; Firman 2011), which is one reason why artificial insemination is required to distinguish the role of paternal genes from maternal effects in generating differences in offspring performance of polyandrous and monandrous females.

In *G. holbrooki*, males barely grow after maturation, suggesting that the smaller size at maturation of polyandrous females' sons might be costly. Indeed, larger males tend to be socially dominant (Bisazza and Marin 1991), have higher insemination success per mating (Head et al. 2015) and are sometimes preferred by females (e.g., Hughes 1985; Bisazza et al. 2001, Kahn et al. 2010, 2012). Crucially, however, when males compete freely there is little evidence that larger body size increases male reproductive success. Indeed, past studies have mainly shown a small male advantage (Bisazza and Marin 1995; Pilastro et al 1997; Head et al. 2017), although studies have also reported a weak advantage for large males (Booksmythe et al. 2016), or no effect of male size (Vega-Trejo et al. 2016). In contrast, there is a clear benefit to earlier maturation if it extends a male's reproductive lifespan by allowing him to start mating earlier in the breeding season. Finally, it should be noted that the growth/size effect of polyandry was sex-specific (only the sons of polyandrous females matured faster). This highlights the broader point that sex-specific differences in the effects of traits on offspring fitness may be important distinctions when it comes to quantifying how different factors affect female fitness.

The importance of disentangling male harassment from polyandry

Some experimental studies report no effect of premating male harassment on female fitness (e.g., Head and Brooks 2006; den Hollander and Gwynne 2009; Galimberti et al. 2000; Gasparini et al. 2012; Helinski and Harrington 2012), others report lower current or lifetime reproductive output (Ojanguren and Magurran 2007; Takahashi and Watanabe 2010; Rossi et al. 2010; Le Galliard et al. 2008; Sakurai and Kasuya 2008; Gay et al. 2009), while still others show beneficial effects on female fitness (Smith and Sargent 2006; Smith 2007; our study). Unfortunately, most studies fail to isolate the effects of premating male harassment on female fitness. This is partly because they have experimental designs that do not control for potential beneficial effects of polyandry when measuring the costs of harassment (i.e., more males harassing equates to more sires); and partly because many studies fail to distinguish effects arising from interacting with males from those due to actual mating (i.e., more male harassment equates to more matings). In those studies that come closest, the level of male harassment is varied while controlling for the cost of mating (i.e., female only mated

once), but the individual effects of polyandry and male harassment are conflated in treatments where increased harassment and increased number of mates is associated with an increased mating rate (den Hollander and Gwynne 2009; Zajitschek et al. 2018). More studies that use our design to simultaneously examine the effects of male harassment and polyandry, while controlling for the actual mating rate are needed to quantify how costly male sexual harassment really is for females.

The value of understanding the costs of male harassment is exemplified by the fact that selection on females to reduce costly male harassment is often invoked as a factor in the evolution of sociality, mate-guarding and mating systems in vertebrates (Clutton-Brock and Parker 1995; Wolff and Macdonald 2004; Muller et al. 2007; Knott et al. 2010; Auclair et al. 2014). This is based on the argument that "convenience polyandry" drives female multiple mating. This claim relies on the untested assumption that male sexual harassment is highly costly to females. Here, we have shown that, controlling for mating, male sexual harassment alone does not always equate to high costs for females. Of course, the relationship between male sexual harassment and female fitness can vary among species, and even populations (Maklakov et al. 2005; Gasparini et al. 2012). Our results in *G. holbrooki* are a reminder that we cannot assume a simple, negative relationship.

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