

## Original Article

The effects of familiarity and mating experience on mate choice in mosquitofish, *Gambusia holbrooki*

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A preference to mate with novel partners has been shown for both males and females in a range of taxa. Preferences for novel mates may result from direct recognition of previous sexual partners, or from other cues that predict this, such as familiarity. Costs and benefits of mating with multiple mates differ for males and females. Despite this, few studies have tested whether the sexes differ in their preferences for novel mates. Here, we investigated whether males and/or females showed preferences for novel mates and whether this differed depending on the type of experience with a familiar mate (i.e., previously allowed to mate or allowed visual and olfactory exposure only) in the eastern mosquitofish (*Gambusia holbrooki*). We show that mosquitofish prefer to associate with novel fish and that there was no significant difference between the sexes in the strength of this preference if the choosing fish had previously had an opportunity to mate. In contrast, males and females that had not recently mated and were familiar due solely to visual and olfactory contact did not have a preference for novel mates. Our results suggest that there are likely to be benefits of mating with multiple partners for both males and females.

**Key words:** Coolidge effect, mate preference, novel mate, remating, sexual selection.

## INTRODUCTION

Mating with multiple partners can be beneficial for both males and females. For females, polyandry can elevate the reproductive value of their offspring because postcopulatory mechanisms bias paternity toward more genetically compatible males (e.g., Tregenza and Wedell 2002) or toward better quality males (meta-analysis: Slatyer et al. 2012). Mating multiply can also increase the likelihood that females receive sufficient sperm to fertilize all their eggs (Pizzari 2002) or that females gain greater access to material resources provided by males, including nuptial gifts and parental care (review: Jennions and Petrie 2000). Although these direct benefits might be achieved by mating repeatedly with a single partner, potential variation in male quality means that these benefits are likely to be greater if females seek new, superior partners. For males, mating with more females allows greater fertilization opportunities and thus increases the total number of offspring sired. This is exemplified by the fact that males almost always have a positive Bateman gradient (i.e., the relationship between offspring number and the number of mates; Bateman 1948). Because the Bateman gradient is generally steeper for males than for females, it is often assumed

that males gain more than females from mating with multiple partners (review: Kokko et al. 2012).

Individuals that prefer novel mates over previous mating partners can increase the likelihood that they mate with multiple partners, rather than repeatedly with the same individual (e.g., Archer and Elgar 1999). Increasing the mating effort invested into obtaining novel mates is a widespread phenomenon often referred to as the “Coolidge effect” (Dewsbury 1981). That is, sexual interest in a previous mating partner declines with each successive mating but is renewed when a novel individual is available (e.g., Koene and Ter Maat 2007; Steiger et al. 2008; Tlachi-Lopez et al. 2012). A decline in sexual interest, and subsequent recovery when exposed to a novel mate, has been measured as changes in latency to mate (e.g., Gershman and Sakaluk 2009), courting effort (e.g., Jordan and Brooks 2010), clutch size (e.g., LaDage et al. 2008), and in ejaculate quality and quantity (Dewsbury 1982; Wedell et al. 2002; Spence et al. 2013). For females, it could even involve postcopulatory processes such as biasing paternity toward a novel mate (e.g., Gershman 2009).

The most common test for a Coolidge effect is to offer an individual a choice between a prior mate and a novel potential mate with otherwise similar mating history (e.g., both are nonvirgins who have recently mated). Preferences for novel over previous mates have been reported

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for both females and males (Archer and Elgar 1999; Kelley et al. 1999; Eakley and Houde 2004; Ivy and Sakaluk 2005; Gershman 2009). To prefer novel mates minimally requires that the choosing sex recognizes and discriminates against familiar individuals with whom they might have mated (Griffiths and Ward 2006; Valero et al. 2009). The likelihood of mating multiply is further increased when individuals can actually recognize novel mates, possibly based on rare phenotypes (Zajitschek et al. 2006; Zajitschek and Brooks 2008) and/or based on the absence of self-referent cues that allow individuals to identify previous mates (Ivy et al. 2005; Steiger et al. 2008).

Strictly speaking, the Coolidge effect refers to a lower sexual response to a previous sexual partner, but sexual interest can also decline simply due to familiarity with an individual (e.g., Zajitschek et al. 2006; Jordan and Brooks 2010). This suggests that in some species, familiarity is a “rule of thumb” to indicate that an individual might have been a previous mate (see Tan et al. 2013). The ability to identify unfamiliar individuals can still offer a reproductive advantage, on average, even if some familiar individuals have not mated (e.g., Kelley et al. 1999).

Experimental studies of mate choice are essential to quantify the relative preference of each sex for novel mates. To date, most studies of the Coolidge effect have examined only a single sex per species (for exceptions, see Zajitschek et al. 2006; Ferkin et al. 2010; Mariette et al. 2010; Tan et al. 2013). Furthermore, taxonomic bias with regard to which sex research has focused on makes evaluation of sex differences in preferences for novel mates difficult. For instance, studies of male choice are more often carried out on small mammals (Dewsbury 1981; Pierce et al. 1992), whereas studies of female choice are more often conducted using insects, fish, and birds (Archer and Elgar 1999; Beguin et al. 2006; Zajitschek and Brooks 2008). Here, we experimentally tested for mating preferences of both sexes for novel versus familiar individuals in the mosquitofish (*Gambusia holbrooki*). We also tested whether the relative size of the potential novel mate had an effect on mate choice. In many fish, females prefer to mate with larger males (Head et al. 2013) and males also prefer to mate with larger females as it is positively correlated with fecundity (Andersson 1994; Casalini et al. 2013). In *G. holbrooki*, there is evidence for both male and female preferences for larger mates (e.g., males: Bisazza et al. 1989; Wong and McCarthy 2009; Mautz and Jennions 2011; Booksmythe et al. 2013 and females: Bisazza et al. 2001; Kahn et al. 2010, 2012).

To measure mating preferences, we initially performed 2-choice trials based on relative association time with a novel or previously encountered fish. We then conducted mating trials where these fish could interact freely. We had 5 aims:

- (1) To test for a mating preference for novel mates (i.e., Coolidge effect).
- (2) To determine if the magnitude of preferences for novel mates depended on whether the previously encountered fish was familiar (i.e., allowed only visual and olfactory exposure; not mated for more than 3 months prior mate exposure) or was a previous mate (i.e., allowed to interact freely; mated within 24 h to mate exposure). We expected the effect to be stronger if the choosing individual had already mated with the familiar fish.
- (3) To test if the effect of familiarity versus actual mating differs between the sexes.
- (4) To test for an effect of the relative size of the novel mate. We predicted a weaker preference for a novel mate if it was smaller than the familiar fish.

- (5) To test whether association time in 2-choice trials predicts how males direct mating effort (copulation attempts) when individuals freely interact.

## METHODS

The mosquitofish used in our study were the offspring of wild-caught fish collected in Sydney (33°48'50.14"S, 150°45'38.75"E) in November 2012 and February 2013. Fish were reared in the laboratory on a 14:10 h photoperiod at 28 °C and fed ad libitum with *Artemia nauplii* and commercial flakes. All the fish used were adults kept in large, same-sex holding tanks once they were old enough to be sexed. Females were male deprived for at least 3 months prior to mate choice tests to ensure that they had identical recent socio-sexual histories.

All females presented to males as potential mates were marked with a small colored dot for visual identification using fluorescent elastomer (Northwest Marine Technology, Shaw Island, WA) injected subcutaneously behind the caudal fin. They had at least 4 days recovery before choice trials. We measured the standard length (SL = snout tip to base of caudal fin) of all fish. Fish from same-sex holding tanks were randomly assigned either as one of the stimulus pair or as a focal test fish. We did not match fish for size (males: 16.8–26.6 mm and females: 23.1–33.2 mm). Each fish was only used once as a potential mate or as a focal test fish whose mating preference was measured. All trios were unique.

### Experimental design

To determine whether male and/or female mosquitofish showed preferences for novel partners, we allowed focal test fish to choose between 2 potential mates: one that they had prior experience with and one that they had no experience with. We applied 2 treatments to fish with whom they had prior experience: mated or familiar. For the mated treatment, the focal fish chose between a stimulus fish that they had previously been allowed to interact freely with (i.e., kept for 24 h together in a 6-L tank) and a stimulus fish that had experienced the same protocol with another fish. For simplicity, we refer to these test fish as “mated.” This is highly probable given the high rate at which males attempt to inseminate females (see Wilson 2005), but we did not directly confirm that mating occurred. For the familiarity treatment, the focal fish chose between a stimulus fish with whom it had previously had visual and olfactory contact (i.e., kept for 24 h together in a 6-L tank separated by a mesh partition) and a stimulus fish that had experienced the same protocol but with another fish. In addition to differing in whether they were mated or familiar, the treatments also differed in mating history. That is, fish from the mated treatment had likely mated in the previous 24 h, whereas fish from the familiar had not mated for at least 3 months prior to trials.

### Association time choice trials

We performed 40 trials per treatment ( $N = 160$ ; 2 sexes and 2 treatments) with observations lasting 10 min (see McLaughlin and Bruce 2001; Simcox et al. 2005; Mariette et al. 2010). After 24 h being kept in either the mated or familiar treatment, fish were individually transferred to separate 1-L tanks and left for 30 min. They were then transferred to the test tank. The test tank was a 16.6-L (38 × 19 × 19 cm) glass aquarium divided into 3 sections: 2 end sections (5 × 19 × 19 cm) held the stimulus fish and a central section (28 × 19 × 19 cm) held the test fish. The sections were

each separated by a removable opaque screen and a mesh screen. A novel fish was randomly assigned to an end compartment at the start of each trial. After a 5-min acclimation period, we removed the opaque screens, so that the fish were in visual and olfactory contact and began the trial. We calculated the test fish's mating preference based on association time (i.e., time spent within 4 cm of the end compartment facing a potential mate). The relative time that the test fish spent at the end compartment housing the novel fish was calculated. For a trial to be included in our analysis, the test fish had to visit both choice zones (18 of 178 trials were discarded for this reason).

### Free-swimming mating trials

To validate the use of association time to measure male mating preferences, we conducted mating trials where test fish were allowed to interact freely with the stimulus fish. Immediately after the choice trial ended, we lifted both mesh screens to allow the 3 fish to interact. We then recorded 1) the male association time with each female (defined as occurring when he was oriented toward a female and within one body length) and 2) the number of mating attempts per female, defined as gonopodium thrusts made after being initially positioned below and slightly behind her. We only conducted mating trials for test males because mosquitofish mating behavior, which consists of males harassing females, makes it difficult to measure female choice when fish freely interact: both males continuously swim alongside the female unless one drives the other away. Males do not court females but instead position themselves below the females in an attempt to transfer sperm through their gonopodium, an organ modified from the anal fin (Bisazza and Marin 1991). We performed 40 trials per treatment ( $N = 80$ ) with each observation lasting 10 min. Data were collected using a handheld event recorder.

To verify the accuracy of data collected directly during the trials, we recorded them with a high-speed video camera. We then compared the data obtained directly with that from analysis of the recordings (collected by an observer blind to the treatment or identity of the novel fish). There was a strong correlation between data collection methods for 1) difference in association time with females ( $r = 0.540$ ,  $P = 0.002$ ,  $N = 30$ ) and 2) number of mating attempts ( $r = 0.792$ ,  $P < 0.001$ ,  $N = 60$ ). Given that these methods of data collection gave similar results, we decided to use data collected directly during the trials as we felt the presence of 3D information made this method more accurate.

### Statistical analysis

We analyzed the relative time the test fish spent with each mate in the choice trials as the proportion of association time spent with the novel fish in a generalized linear model (GLM) with quasi-binomial error using the `cbind` function in R 3.0.2 software (R Development Core Team 2012; i.e., including information on the total amount of time each fish spent in association with potential mates). We included sex, treatment, and the interaction between these factors as fixed factors in the model. We also included the size difference between the potential mates (novel minus familiar or mated) as a covariate, as size is known to be important in mate choice in mosquitofish. We did not include any other 2- or 3-way interaction terms in the model because we decided a priori that these were not the aim of this study (their inclusion does not, however, change the key findings we report). We included which side the novel fish was on to control for any inadvertent side bias. To directly test for a

preference for novel mates in each treatment by each sex, we then followed this analysis with 4 separate GLM models (as above) that only included size difference as a predictor. Here, we were interested in the estimate of the intercept (i.e., when stimulus fish are the same size, do focal fish show a preference for a novel partner?). Given the clear prediction, we used 1-tailed tests (i.e., we predicted that the intercept was greater than 0). An intercept of 0 corresponds to 50% of the time spent with each mate ( $\ln(p/[1-p])$ , where  $p$  = proportion of time with the novel mate). If the trend was in the opposite direction, we report the 2-tailed  $P$  value. Unless otherwise noted, 2-tailed  $P$  values are reported.

To examine male preferences in the free-swimming mating trials, we used the proportion of copulation attempts directed at the novel female as the dependant variable in a GLM with quasi-binomial error using the `cbind` function. Treatment, size difference, and their interaction were included as fixed factors in the model. We excluded 12 of 80 trials because the focal male did not attempt to mate with either female so they were uninformative. We again followed this analysis with 2 complimentary GLM models with size difference as the sole predictor specifically to test for a preference for novel partners within each treatment by testing whether the intercept was significantly greater than 0 (description above).

Finally, we tested whether we could predict the relative number of male attempts directed at the novel female in the free-swimming mating trials using the relative time spent associating with her during the choice trials. We used the proportion of the male attempts directed at the novel female as the dependant variable in a GLM with quasi-binomial error using the `cbind` function. The treatment, the proportion of association time spent with the novel female during the choice trial, and their interaction were included in the model as fixed factors.

## RESULTS

### Association time choice trials

There was no significant difference between males and females in the proportion of time spent in association with the novel fish. There was also no interaction between the sex of the test fish and whether or not the previously encountered fish was a familiar or previously mated fish (i.e., familiar/mated treatment). However, test fish spent significantly more time associating with a novel fish when the previously encountered fish was a former mate, rather than only a familiar fish (Table 1). Both males and females spent significantly more than 50% of their time in association with a novel male in the mated treatment trials (GLM<sub>males mated</sub> = 0.639 = 65.5%,  $t_{(39)} = 2.676$ ,  $P_{1\text{-tail}} = 0.006$ ; GLM<sub>females mated</sub> = 0.481 = 61.8%,  $t_{(39)} = 2.253$ ,  $P_{1\text{-tail}} = 0.015$ ). The 2 intercepts (i.e., estimates of the proportion of time spent with a novel mate) did not differ significantly from each other:  $t_{78} = 0.699$ ,  $P = 0.487$ , Figure 1. There was, however, no preference for novel mates in the familiar treatment (GLM<sub>males familiar</sub> = -0.100 = 47.5%,  $t_{(39)} = -0.363$ ,  $P = 0.719$ ; GLM<sub>females familiar</sub> = -0.153 = 46.2%,  $t_{(39)} = -0.673$ ,  $P = 0.505$ ; Figure 1).

### Free-swimming mating trials

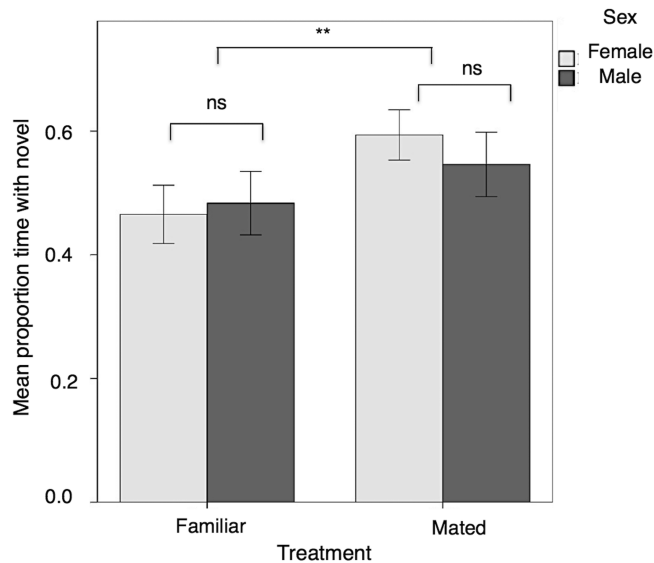
We found a highly positive correlation between the relative time that a male spent close to the novel female during the mating trial and the proportion of his mating attempts directed toward the novel female ( $r = 0.811$ ,  $N = 68$ ,  $P = 0.01$ ). Mating attempts are a direct measure of male sexual interest, so we have only analyzed

**Table 1**

**Results of GLMs (quasi-binomial error) for the response variables: proportion of time with novel fish in choice trials and proportion of male mating attempts directed at the novel fish during mating trials**

Response variable and factors	df	F	P
Proportion of time with novel fish			
Sex	1,158	0.052	0.820
Treatment	1,157	9.942	<b>0.001</b>
Size difference	1,156	2.160	0.144
Side	1,155	4.455	<b>0.036</b>
Sex × treatment	1,154	0.669	0.415
Proportion of male attempts directed at novel fish			
Treatment	1,66	0.105	0.748
Size difference	1,65	14.427	<b>&lt;0.001</b>
Treatment × size difference	1,64	11.934	<b>0.001</b>

The main effects are sex (male/female), treatment (mated/familiar), size (novel—familiar or mated), and side (novel fish on the left or right). df, degrees of freedom. Bold values represent significant values.

**Figure 1**

Mean  $\pm$  SE proportion of association time spent with the novel individual given the previously encountered fish was assigned to the mating or familiar treatment. Significant differences ( $P < 0.05$ ) are represented by \*\*. Note that these are raw means that do not account for size difference between the novel and familiar fish and that treat each data point equally (i.e., unlike the GLM in Table 1, there is no weighting by the total time the focal fish spent in association with potential mates).

this measure of male choice. Unexpectedly, the effect of relative female size on the proportion of attempts directed at the novel female depended on whether or not the male had previously had the opportunity to mate with her (Table 1). Males that were only familiar with the previously encountered female, but had not had a chance to mate with her, were unaffected by the female size difference ( $F_{1,32} = 0.001$ ,  $P = 0.982$ ). In contrast, if there had previously been an opportunity to mate, then the male was more likely to direct his mating attempts toward the larger of the 2 females ( $F_{1,34} = 6.509$ ,  $P = 0.016$ ) (Figure 2). We did not find a significant preference for novel mates in either treatment although there was a marginally nonsignificant trend in the mated treatment (GLM<sub>mated</sub> = 0.246 = 56.1%,  $t_{(34)} = 1.606$ ,  $P_{1\text{-tail}} = 0.059$ ; GLM<sub>familiar</sub> = 0.010 = 52.5%,  $t_{(32)} = 0.060$ ,  $P_{1\text{-tail}} = 0.476$ ).

## Predicting male courtship by association time

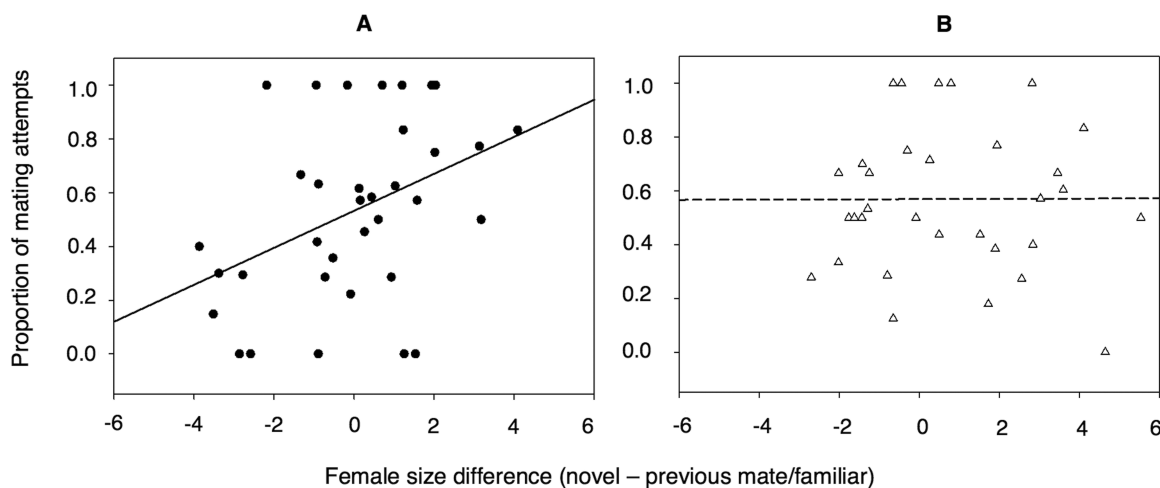
The proportion of time a male spent in association with the novel female during the choice trials predicted the proportion of his mating attempts subsequently directed at her during the free-swimming mating trials ( $F_{1,66} = 16.386$ ,  $P < 0.001$ ). There was no difference between the familiar and mated treatments in the strength of this relationship ( $F_{1,64} = 0.026$ ,  $P = 0.873$ ; Figure 3). Association time in 2-choice trials was, therefore, a good general predictor of actual male mate choice as measured by mating attempts.

## DISCUSSION

We found that, under certain conditions, mosquitofish (*G. holbrooki*) exhibited a significant preference for novel individuals (i.e., Coolidge effect). Contrary to expectations based on a stronger relationship between the number of mates and reproductive success in males than females (i.e., males have a steeper Bateman gradient; Kokko et al. 2012), there was no significant differences between the sexes in the strength of the observed Coolidge effect (Table 1). Others have, however, argued that higher costs per mating for females than males and ongoing benefits of repeated mating with the same female when there is sperm competition could actually generate stronger selection on females than males to discriminate against previous mates (see Mariette et al. 2010). Thus, the relationship between the number of mates and offspring number alone might not account for the Coolidge effect. It is worth noting that Bateman gradients are often short-term estimates of the fitness returns of mating and, therefore, ignore any longer-term costs associated with an increased number of mates (e.g., future effects on mortality, fecundity, or sexual attractiveness; Kokko et al. 2012).

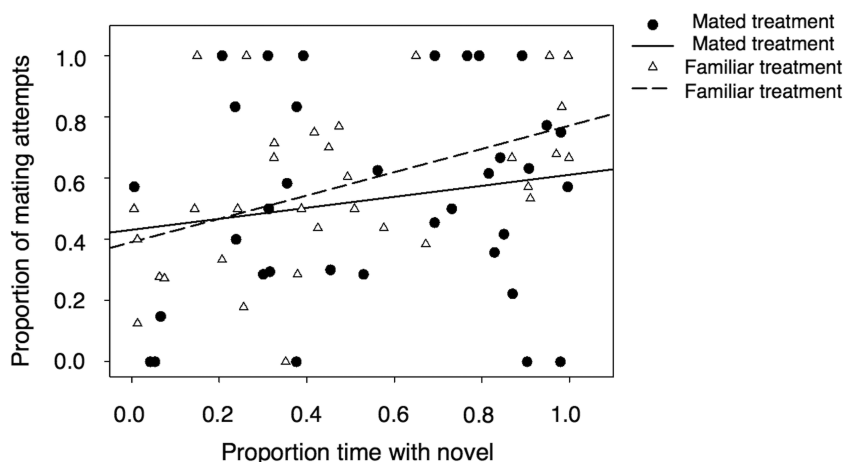
In mosquitofish, whether males or females exhibited a preference for novel mates depended on whether the alternate mate was an individual that the test fish had previously encountered (i.e., familiar treatment) or one with whom they had actually had the opportunity to mate (i.e., mated treatment). When the alternate fish was from the familiar treatment, neither sex showed a significant preference for a novel partner. This result is consistent with our prediction that the Coolidge effect should be stronger when mating had occurred. Familiarity alone should not affect the value of a potential mate.

Alternatively, the difference in the preference for novel mates seen between our mated and familiar treatments may be due to differences in the recent mating history of test (and stimulus) fish. All fish in the mating treatment had recently mated, but in the familiar treatment, they had not. In some species, virgin females are less choosy than mated females (e.g., Pitcher et al. 2003), and likewise, males with smaller sperm reserves following a recent mating might be more choosy than those with full reserves (Bukowski et al. 2001; Bateman and Ferguson 2004). The lack of preference for novel partners in our familiar treatment might therefore have arisen because focal fish had not mated in the previous 3 months and were therefore generally less choosy than those in the mated treatment. To our knowledge, only a few studies have teased apart the effects of familiarity and recent mating history on mate choice for novel mates (e.g., Zajitschek et al. 2006; Tan et al. 2013). Such tests are necessary to confirm with certainty that there is a direct effect of experience type (i.e., familiar only vs. mate) on preferences for novel individuals. It should be noted, however, that the mating history of potential mates was identical within each treatment, so there is still clear evidence for a Coolidge effect in the mating treatment.



**Figure 2**

The association between the female size difference (novel – previous mate/familiar mate) and the proportion of male mating attempts directed at the novel female during mating trials. (A) Mated treatment. (B) Familiar treatment. Note that the regression line drawn is based on treating each data point equally (i.e., unlike the GLM in Table 1, there is no weighting by the total number of times that focal males attempted to mate).



**Figure 3**

The relationship between the proportion of time spent with the novel female during choice trials and the proportion of male mating attempts directed at her during mating trials. Note that the regression lines shown are based on treating each data point equally (i.e., unlike the GLM in the text, there is no weighting by the total number of times that focal males attempted to mate).

### A comparison with guppies

Poeciliid fish are the subject of intense investigation by those studying sexual selection (Evans et al. 2011), but relatively few studies have investigated the Coolidge effect. Most of these studies are on guppies (*Poecilia reticulata*), so they are our main source of comparison to *G. holbrooki*. Although the mating systems of these 2 species differ (male guppies court and coerce females, whereas male mosquitofish only coerce females), several studies have shown that female mosquitofish are able to exert mate choice by preferentially associating with certain males and thus increasing the likelihood of insemination by these males (e.g., Bisazza et al. 2001; Pilastro et al. 2003; Kahn et al. 2010, 2012).

In guppies, females generally prefer novel over familiar males (e.g., Zajitschek et al. 2006; Mariette et al. 2010). This is consistent with a trend for female guppies to prefer males with phenotypes that they are unlikely to have encountered previously (“rare male effect,” see Zajitschek and Brooks 2008; Hughes et al. 2013). This mating preference has been attributed to inbreeding avoidance

because unfamiliar males with unusual color patterns are more likely to be unrelated (Kelley et al. 1999; Mariette et al. 2006; Zajitschek et al. 2009). Our result that a preference for novel partners only exists when focal fish have mated with the familiar fish suggests that inbreeding avoidance is unlikely to be the driver of preferences for novel partners in mosquitofish. Furthermore, the “rare male effect” may be less likely to contribute to the patterns we see here than is the case in guppies because guppies show extreme color pattern polymorphism, whereas mosquitofish do not.

Although we have documented male and female preferences for novel mates in mosquitofish, the adaptive significance of these preferences is unclear. For males, the benefits of mating with multiple partners are straightforward—more mates means more offspring. For females, reasons for preferring novel mates are less clear. First, females may gain indirect benefits of mating with multiple partners if it allows females to bias paternity toward males that sire higher quality offspring (e.g., Evans and Magurran 2000). However, general evidence for this benefit is weak (meta-analysis: Slatyer et al.

2012). Second, females may gain a fertilization benefit. This is plausible if male sterility is common or if genetic incompatibility between males and females leads to a failure to fertilize eggs (Pizzari 2002; Wedell et al. 2002). Assessing whether the patterns we see here are driven by such fertility benefits is difficult as there is little information available about variation in male fertility or genetic incompatibility in mosquitofish. As such, this remains a potentially important form of selection that deserves further investigation. Third, females may avoid males that have previously harassed them (Bisazza et al. 2001; McLaughlin and Bruce 2001). This could explain why females only showed a preference for novel males in our mated treatment where fish were allowed to fully interact as opposed to the familiar treatment where males could not engage in coercive behavior. Finally, direct interactions between fish (i.e., during the mating treatment) may provide additional cues that increase the ease with which a female can recognize familiar males (Hughes et al. 1999). If true, females in the familiar treatment may have been less capable of discriminating between novel and familiar males than those in the mated treatment. In the choice trials, both males and females showed a significant preference for associating with a novel mate, but only if they had an opportunity to mate with the previously encountered fish. In the free-swimming mating trials, however, there was only a nonsignificant tendency for males to attempt to mate with the novel female ( $P = 0.059$  from the GLM).

In guppies, there is contradictory evidence as to whether males prefer novel, unfamiliar females. For example, no such preference was reported by Zajitschek et al. (2006), but one was reported by Kelley et al. (1999), Mariette et al. (2010), and Jordan and Brooks (2010). Mariette et al. (2010) showed, however, that females showed a stronger preference for unfamiliar mates than males did and suggested that this difference could result from indirect effects of female behavior (e.g., preferences or receptivity) on male behavior. Indirect effects of female behavior on male behavior may also explain differences in male preferences for novel mates that we see here between trial types, as male preference for novel females was lower when males and females were allowed to interact freely.

In our study, one unexpected finding from the free-swimming mating trials was that males that had previously had an opportunity to mate with the familiar female directed a significantly higher proportion of their mating attempts to whichever of the 2 females was the larger. In contrast, there was no effect of relative female size on the proportion of mating attempts directed at each female by males that had only had visual and chemical contact with the familiar female (Figure 2). Males then tried to mate with larger females even when they had previously mated with them. It is possible that males did not prefer mating with novel females, but only bigger females, or even that mating with any female takes precedence over any other discrimination (Sievers and Magurran 2011).

### Validating association choice tests

Few studies of mate choice based on association times in setups equivalent to our choice trials have validated that association time during choice trials predicts mating behavior when fish can freely interact (review: Jeswiet and Godin 2011). We found that the proportion of time males spent with a novel female during choice trials was significantly positively related to the proportion of mating attempts directed at the same female during mating trials when fish could freely interact. Our study is, therefore, in agreement with studies of other Poeciliid fish in showing that association time is a reasonable proxy for actual mating behavior (guppies: Jeswiet and Godin 2011; swordtails: Walling et al. 2010). This strengthens the

interpretation of previous studies of male choice in mosquitofish based on association time (e.g., Bisazza et al. 1989; Mautz and Jennions 2011; Callander et al. 2012; Booksmythe et al. 2013).

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