Original Article

An experimental test to separate the effects of male age and mating history on female mate choice

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Should females prefer older males as mates? Male survival to old age might indicate the presence of fitness-enhancing genes that increase offspring fitness. However, many correlational studies show that mating with older males can lower female fecundity and even reduce offspring fitness due to epigenetic or germline mutation effects. One problem in quantifying female choice based on male age is that age is usually confounded with mating history. This begs a question: Do females choose males based on their age or their mating history? The answer requires an experimental approach, but few such studies exist. Here, we test if experimentally induced variation in the mating history of old and young males (12-week difference in postmaturity age) affects female choice in the eastern mosquitofish (*Gambusia holbrooki*). To vary mating history, adult males were either allowed to freely mate with females for 3 weeks or they only had visual contact with females. Immediately thereafter, we ran four-choice mating trials, using association time, to test the effects of male age and mating history (2 × 2 design) on male attractiveness. Females did not show a clear preference for males based on either characteristic. This was not due to a lack of female choice: females spent significantly more time with larger males. In addition, female choice was significantly repeatable across four trials: twice as a virgin and twice as a nonvirgin. Finally, female mating status (virgin or nonvirgin) did not affect her choice of mate, although virgin females spent significantly more time associating with test males.

Key words: aging, mate choice, mating experience, mosquitofish, repeatability, senescence.

INTRODUCTION

Female mate choice occurs when females mate nonrandomly based on variation in male traits, such as the expression of exaggerated sexual traits, the resources they offer females, and their social status (Edward 2014; Ronald et al. 2018). Being choosy can provide females with direct benefits, for example, material resources (e.g., nuptial gifts and nesting sites), and/or genetic benefits that increase mean offspring fitness (Andersson 1994; Brooks and Kemp 2001; Radwan 2003; Kuijper et al. 2012). There are, however, many factors that affect the costs and/or benefits of female choice, including a female's age, her mating status, and the relative availability of mates. These factors often result in variation among females in their mate choice decisions (Jennions and Petrie 1997; Jennions and Petrie 2000; Brooks and Endler 2001; Narraway et al. 2010; Kelly 2018).

Among the factors that affect male attractiveness, male age has been repeatedly shown to be important (Bonduriansky et al. 2008; Roach and Carey 2014; Scauzillo and Ferkin 2019).

Earlier theoretical models predicted that females should choose older males, as prolonged survival could be an indicator of fitness-enhancing genes that will be inherited by their offspring (reviews: Kokko and Lindstrom 1996; Kokko 1998). That is, being old is a signal of the quality of a male with respect to traits that increase lifespan and, by extension, net fitness (Brooks and Kemp 2001; Radwan 2003). This prediction has been supported by numerous empirical studies that show females prefer older males. This occurs in, for example, humans (Conroy-Beam and Buss 2019), birds (great bustards: Alonso et al. 2010; brown-headed cowbirds: Zipple et al. 2020), and insects (e.g., fruitflies: Verspoor et al. 2015; wild crickets: Rodríguez-Muñoz et al. 2019; broad-horned flour beetles: Okada et al. 2020). However, other theoretical models predict the evolution of female preferences for younger males because the probability of reduced fertility or lower offspring viability can increase with male age (Beck and Powell 2000; Beck and Promislow 2007). Such a decline in male performance could occur because deleterious germline mutations accumulate over time so that mating with older males can decrease both fertilization success, hence realized female fecundity, and offspring viability (Brooks and Garratt 2017; Evans

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et al. 2019; Wylde et al. 2019). These costs should favor a female preference for younger males or at least against mating with much older males, and many studies have reported evidence for this type of preference. For example, females prefer younger males in both a moth and in roe deer, (Lai et al. 2019; Vanpé et al. 2019) and they lay twice as many eggs after mating with younger males in zebrafish (Kanuga et al. 2011). These opposing theoretical predictions generate uncertainty about the circumstances under which older or younger males will be preferred as mates. To further illustrate the complexity of the situation, some studies have found no detectable effect of male age on female mate choice (e.g., guppies: Gasparini 2010). This could be because the cost of assessing male age is generally high or simply due to an inability of females to detect male age because there are no reliable cues (Beck and Promislow 2007; Johnson and Gemmell 2012). Species where male age has no effect on female choice also raise questions about confounding factors that might obscure the expression of female mating preferences for older or younger males.

One key factor that is often confounded with a male's age is his mating history, which can also affect female fitness (Arnqvist and Nilsson 2000; Perez-Staples et al. 2010). After all, older males have usually mated and bred more often than younger males (Monaghan and Metcalfe 2019). Females might, therefore, prefer males with a more extensive mating history rather than older males per se because of the potential advantages of mating with a more sexually experienced partner. For example, in nereidid polychaetes, females choose males with a more extensive mating history who provide greater parental care (Fletcher et al. 2009). In contrast, males that have a longer mating history might become sperm limited, and mating with them could lower fertilization success such that females benefit by discriminating against them. For instance, female guppies prefer virgin males (Scarponi et al. 2015; Scarponi and Godin 2018). As natural variation in mating history is confounded with male age, it is unclear which of these two factors is more important in driving female mate choice. If older males are more attractive, is it because of their age or longer mating history, which is often correlated with their age? Ideally, we need to experimentally tease apart male mating history and age to determine if they have independent main effects and possibly even an interacting effect on female mate choice. In most observational studies, mating history is confounded with age, precluding us from determining the main effect of age. To date, very few studies have experimentally disentangled the effects of male mating history and age (but see: Jones and Elgar 2004; Perez-Staples et al. 2010). Here, we quantify the causal effects of male age and mating history on female choice in eastern mosquitofish (Gambusia holbrooki) by experimentally manipulating the mating history of young and old males.

In addition to disentangling the effect of male age and mating history when conducting mate choice experiments, it is necessary to consider other sources of variation in female choice. As noted earlier, female choice often depends on her current mating status (Jennions and Petrie 2000; Brooks and Endler 2001; Travers et al. 2016; Rivers and DuVal 2019). For instance, nonvirgin female guppies tend to be choosier than virgins (Pitcher et al. 2003), while, in crayfish, nonvirgin females are less choosy (Mellan et al. 2014). It is, therefore, recommended to test for any effect of female mating status on mate choice decisions (Kodric-Brown and Nicoletto 2001; Bell et al. 2009; Kelly 2018), including choice based on male age. Finally, while many studies have documented female mating preferences in mate choice trials, the repeatability of choice is often

unknown. Testing for repeatability is, however, valuable because significant repeatability offers confirmation that females are exhibiting genuine choice (Forstmeier and Birkhead 2004; Bell et al. 2009). Such tests are often neglected when running mate choice experiments, although some studies on fish have reported repeatable mate choice (e.g., annual killifish: Passos et al. 2013; guppies: Godin et al. 2005; Godin and Auld 2013). In addition, such tests can be used to determine whether individual variation in preferences persists despite a change in other parameters (e.g., a female's mating status).

Here, we ran female choice experiments using eastern mosquitofish (*G. holbrooki*), a species with strong sexual dimorphism and coercive mating (Bisazza and Marin 1995). Although *G. holbooki* might seem an unusual species with which to investigate female choice for male age and mating experience, there are several reasons why it is an appropriate option. First, female choice in *G. holbrooki* readily arises through female association preferences: by spending more time with certain males, females increase the likelihood that these males will coercively mate with them (Vega-Trejo et al. 2014). Second, although there are no obvious direct benefits of choosing older or more experienced males, there is no a priori reason to preclude genetic benefits of mating with older males. In addition, male mating experience affects sperm traits that could affect realized female fecundity (Vega-Trejo et al. 2019; Iglesias Carrasco, Fox, et al. 2019; Iglesias Carrasco, Fox, Vega-Trejo et al. 2019). Third, if we only study species with parental care (or obvious direct benefits of female choice) when testing for mating preferences based on male age, we can never run a comparative analysis to determine which species-level factors predict the presence of female choice for older/younger males. Finally, unlike birds and mammals, which are popular subjects for the study of mating preferences for older males, it is logistically easy to experimentally separate the effects of male age and mating experience.

In the laboratory, we bred up cohorts of virgin males 12 weeks apart to generate our "old" and "young" males. These males were then given different levels of access to mates. One group of males had full mating access to a female for a period of 3 weeks (with females rotated between tanks weekly to maintain male interest in mating). These became our "old/mated" and "young/mated" males. The second group had visual contact with females (to ensure that males were sexually primed) but were separated by a mesh barrier and, therefore, unable to mate. These became our "old/naïve" and "young/naïve" males. Then, controlling for female age and size, we tested for potential effects of male age and mating history on female mate choice. We tested virgin females in two consecutive four-choice trials. We then allowed each female to mate and, the following day, retested their mate preferences in another two sets of four-choice trials. We then tested for repeatability of mate choice within and across days. We had three study questions: 1) Do male age and/or mating history affect female mate choice? 2) Does a female's mating status influence her mate choice decision? 3) Is female mate choice repeatable both within and across days? Based on previous studies showing that sperm traits and body condition (growth and immunocompetence) are negatively affected by male age and mating experience (Iglesias-Carrasco, Fox, et al. 2019; Vega-Trejo et al. 2019) and that mating experience has no positive effect on male mating ability (Iglesias-Carrasco, Fox, et al. 2019), we predicted that females would prefer young/naïve males because they are healthier and more likely to have viable sperm. We also hypothesized that nonvirgin females would be choosier (Pitcher et al. 2003).

METHODS

Origin and maintenance of fish

To examine the effects of male age and mating history on female mate choice in *G. holbrooki*, we bred "young" and "old" males in the laboratory and provided them with two different mating treatments. All fish were maintained in single-sex 90-L tanks at densities of <50 individuals per tank and fed ad libitum twice daily, with commercial fish flakes for the first feed and *Artemia salina* nauplii for the second feed. They were kept under a 14:10 h photoperiod at 28 ± 1 °C.

To produce "old" males, a total of 400 fish (200 males and 200 females) were mixed together in large breeding tanks (90 L at densities of <50 individuals per tank) and held for 18 days to mate. Females were then transferred to individual 1-L tanks containing a mesh barrier at one end to create a refuge for any fry produced. These tanks were checked twice daily for the presence of offspring. For each brood produced, a maximum of 10 individuals were selected at random and transferred to 90-L stock aquaria up to a density of 50 individuals per tank. To minimize the within-group age difference of experimental males, offspring were only collected over a fixed, 15 days period, after which no more individuals were added to stock aquaria. The maximum within-cohort age difference was, therefore, 15 days, although most individuals were born within 10 days of each other. Then, 12 weeks later, using a different set of 400 adult males and females, we reared a second set of offspring to produce "young" males following the procedure described above. Offspring were collected from gravid females, held in 90-L stock aquaria, again with a maximum within-cohort age difference of 15 days.

From 4 weeks of age onward, offspring were inspected three times per week to determine their sex, and immature males were removed to male-only tanks to ensure that they remained virgins. Sexually mature males were identified by the presence of a fully formed gonopodium with distal spines, and females by the presence of a visible gravid spot on the ventral flank of the body in line with the anal fin. As soon as males reached sexual maturity, they were transferred to individual 1-L tanks. Tanks were filled sequentially so that time to maturity could be matched between "young" and "old" males at the later experimental stage. On average, "old" males were 12–13 weeks older in postmaturity age than "young" males.

In addition to creating two sets of males that differed in their postmaturation age, we included an experimental treatment based on whether or not a male had directly interacted, hence mated, with a female. "Naive" males were kept in visual contact with a female but with a mesh barrier separating them to prevent any physical contact; "mated" males were housed with a female so that they could interact and mate freely. Equal numbers of "young" and "old" males were assigned to each mating treatment for 3 weeks to create four treatments ("old/mated"; "young/mated"; "old/naive"; and "young/naive"; *n* [62 × 4 treatments] = 248 males). Prior to entering the mating treatment as a block, four males (one of each type) were randomly marked with a different-colored elastomer tag (Northwest Marine Technology, Shaw Island, WA) for identification to create a block of four males. Males were anesthetized in an ice-water slurry and then injected subcutaneously in the dorsal musculature just below the posterior end of the dorsal fin.

The females we used as stimuli or mates in this part of the experiment were collected from the wild 3 months prior to the start of the experiment and held in female-only aquaria under the conditions described above. Each test male was housed in a 7-L tank (17 × 28 × 15 cm) with a female. The females were rotated among tanks every 7 days to maintain the sexual interest of the males (to avoid the "Coolidge effect"; see: Vega-Trejo et al. 2014). After 3 weeks in their assigned mating treatment, males were isolated for 5 days to replenish their sperm before being used in a female choice trial. Hence, during mate choice trials, the old and young males were approximately 4 and 16 weeks postmaturity. We decided this age difference was relevant to test for a male age effect on female mate choice as a recent study on mosquitofish found a significant decline in sperm traits in males that were 14 weeks postmaturity (Vega-Trejo et al. 2019).

The females we used in the choice trials were the offspring of wild-caught mothers that were born and raised in the laboratory without any physical contact with males. On the first day of the mate choice trial, we used these virgin females in two mate choice assays. We then allowed them to mate before we retested them on the following day (details below).

Measurement of relative attractiveness of males in a mate choice setting

Female preference for males of each treatment type (old/mated, young/mated, old/naive, and young/naive) was tested in four-choice trials (following Booksmythe et al. 2013) over two consecutive days. Each test female saw the same block of four males in all four trials.

On the first day, a virgin focal female and a random nonfocal female were placed in a clear plastic cylinder in the center of a square tank (45- × 45- × 10-cm deep) with four corner compartments, each of which held one male (Figure 1). Each corner compartment was separated from the central compartment by a removable opaque screen. Each wall of the central compartment also had a protruding opaque screen to reduce visual contact
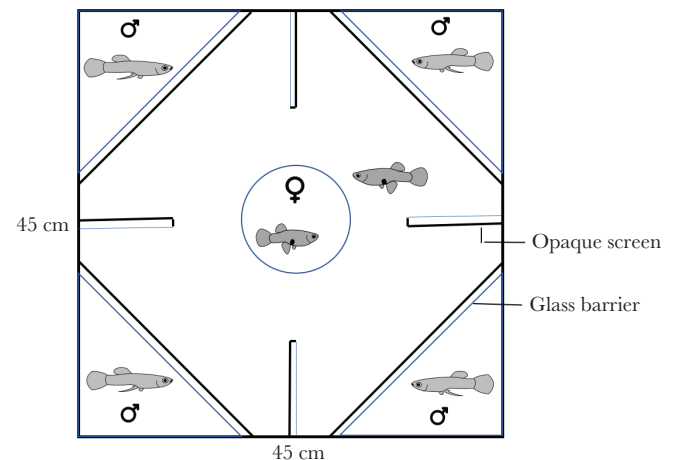
Figure 1

Schematic diagram of apparatus used to test female mate choice in *Gambusia holbrooki*. There are four compartments, one at each corner of the glass tank, containing a male from each of the four treatments: old mated, young mated, old naive, and young naïve. The side barriers at 90° to the walls limited visual contact among males. The cylinder in the middle contains two females. Initially, both females are acclimated in the cylinder. During the mate choice trial, the nonfocal female remains in the cylinder as a potential schooling partner, so the focal female has the option of schooling with a conspecific without having to associate with any of the males. The external walls of the tank were covered in black corflute to prevent fish being disturbed by any external stimuli.

between males during the trial. After a 5-min acclimation period, the opaque barriers in the corners were removed and the focal female was released from the cylinder. The nonfocal female remained in the plastic cylinder so that the focal female did not have to approach a male if she wished simply to school with a conspecific. We then recorded the association time of the focal female with each of the four males for 15 min (following Booksmythe et al. 2013). Her preference for each male was quantified as the time spent <6 cm from his compartment (a distance equivalent to being within approximately two body lengths of the male). The mating system of *G. holbrooki* is such that associating with a male is equivalent to female choice as males try to mate with any nearby female (Vega-Trejo et al. 2014). At the end of the trial, the male compartments were again covered with opaque screens and the fish were given a rest period of 5 min. Then, the screens were again removed from the male compartments and the focal female released from her cylinder and we recorded her movements for a further 15 min. After the second trial, we removed the nonfocal female from the tank and the focal female was then allowed to interact freely with the four males for 40 min, during which time males were able to mate with the female, and we assume that some matings occurred. After that, males and females were returned to their individual tanks. Then, on the following day, a third and fourth choice trial was run (as on the first day) to test whether change in a female's mating status from virgin to mated/nonvirgin affected her mate choice. It should be noted that, after each trial, the choice tank was rotated 90°, so the males were displaced from their original position to reduce any positional bias of females. All trials were scored live by a single observer (blind to male treatment type) and filmed so that the observations could later be independently verified. We initially ran trials on 62 blocks of males. However, if a female only spent time with one male and did not inspect at least one other male, we discarded that block from the trial. This left us with 55 blocks (*n* [55 × 4 treatments] = 220 males).

Statistical analysis

We analyzed the time each focal female spent per trial associating with males from each treatment in a general linear mixed-effect model using the R package glmmTMB (Brooks et al. 2017). Male age, male mating history, female mating status, and their two-way and three-way interactions were treated as fixed factors. We excluded nonsignificant interactions from the final model to test for main effects (all interactions were nonsignificant; see Results; Supplementary Tables S1 and S2). In addition, male body size was included as a covariate (mean-centered per block). In a second model, we included both mean male body size per block and the difference between the absolute and mean body size per block as two continuous covariates to disentangle between- and within-block effects (see Van de Pol and Wright 2009). We added block ID (which is equivalent to female ID), trial order, and trial order nested in block ID as random factors (as there were four data points per test female per trial). The time spent by a female with a male was analogous to count data (number of seconds with a male). It should also be noted that the time spent with males does not sum to the total trial time because females could choose to associate with the nonfocal female or simply roam the tank. No female spent more than 75% of her time with a single male. Consequently, the data could be modeled with a distribution that does not have a maximum or require compositional statistics. The distribution of the data was right skewed and zero inflated. We, therefore, assumed a negative-binomial distribution of the residuals to account for

overdispersion and zero inflation. This provided a good fit to the data (see Supplementary Figure S1).

Following reviewers' comments, we also ran an exploratory model including the color of the marker tag as a fixed factor in our original model. The strength of the effects of other predictors remained similar, despite the color of the tag having a significant effect on female choice (see Supplementary Table S3 for details).

To quantify the repeatability of female mate choice, we ran a multivariate linear mixed model with four response variables: each response variable corresponded to the time spent with a male in a given trial. We fitted block as a random effect for each of the response variables, with a Gaussian distribution for the residuals and extracted the residual variance–covariance to compute correlations across trials in the R package MCMCglmm (Hadfield 2010). We set the alpha significance level at 0.05. All models were run in R Version 3.6.0 (R Core Team 2019).

RESULTS

Male age and male mating history

Neither male age nor male mating history had a significant effect on female choice (Table 1; Figure 2). There was no significant interaction between male age and male mating history ($\chi2 = 0.016$, degrees of freedom [df] = 1, $P = 0.900$; see Supplementary Table S1). The differences in predicted time spent with males of different ages and mating experiences were small (<8.3 s) and not significant (Figure 2). In addition, female mating status did not significantly moderate the effect of male age or male mating history on her choice of mate (all interactions, $P > 0.1$; see Supplementary Table S2). When we excluded interactions and only treated female mating status as an additive effect, there was weak evidence that nonvirgin females spent less time with males than virgins ($\chi^2 = 3.897$, df = 1, $P = 0.048$; Table 1).

Male body size

Controlling for any effects of male age and mating history, there was a strong effect of male body size on female mate choice (Tables 1 and 2; Figure 3). Females spent significantly more time with relatively larger males within each block ($\chi^2 = 7.460$, df = 1, $P = 0.005$). In addition, there was a significant effect of variation in mean male size across blocks: females spent more time with males in blocks where the mean absolute size of males was greater ($\chi^2 = 8.545$, df = 1, $P = 0.003$). The within-block and between-block effects of body size were very similar (slopes: 0.082 ± 0.029 vs. 0.091 ± 0.031; slope = extra seconds spent with a male per 1-mm increase in male body size).

Table 1

Parameter estimates and Anova test statistics (type III Wald chi-square test) values for the effect of male age, male mating history, and female mating status on female mate choice in eastern mosquitofish (*Gambusia holbrooki*). The bold font indicates significance at the 0.05 level

Predictor	Estimate	SE	$\chi2$ (df = 1)	*P*
(Intercept)	4.154	0.087	2263.634	**<0.00001**
Age (young)	0.029	0.073	0.164	0.686
Mating history (naïve)	0.094	0.072	1.687	0.194
Female status (nonvirgin)	−0.142	0.072	3.897	**0.048**
Mean-centered body size	0.087	0.021	16.659	**<0.00005**
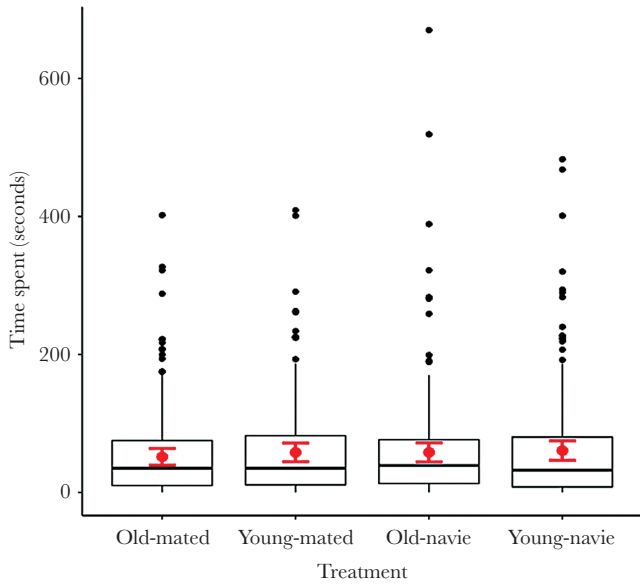
Figure 2
The effect of male age and mating history on female mate choice in *Gambusia holbrooki* (n = 4 × 55 = 220). Box plots show median (horizontal line) and interquartile range of the raw data. Mean and error bars in red represent the mean and 95% confidence interval values from the mixed model prediction.

Table 2
Parameter estimates and Anova test statistics (type III Wald chi-square test) values for the effect of male body size (within and between blocks) on female mate choice in eastern mosquitofish (*Gambusia holbrooki*). The other predictors are not shown, but the strength of the effects are almost identical to those in Table 1. The bold font indicates significance at the 0.05 level

| Predictor	Estimate	SE	χ^2 (df = 1)	P
Relative to average body size in block (within-block effect)	0.082	0.029	7.460	**0.005**
Mean body size (among-block effect)	0.091	0.031	8.545	**0.003**

Repeatability of female choice

The correlations obtained from the multivariate model showed consistency in the ranking of time a female spent with a given male between the first and second trial on a given day and the same trial (first or second) on different days (Table 3). Unexpectedly, however, the correlations were stronger across days (r = 0.209, P = 0.002 for trial 1; r = 0.553, P < 0.001 for trial 2) than within a day (r = 0.169, P = 0.022 for day 1; r = 0.194, P = 0.016 for day 2). The correlations were small and not statistically significant between the first and second trial on different days (r = 0.021, P = 0.916 for day 1 trial 1/day 2 trial 2; r = 0.025, P = 0.404 for day 1 trial 2/day 2 trial 1).

DISCUSSION

Male age and mating history

We experimentally disentangled the effects of male age and male mating history on female mate choice in the mosquitofish
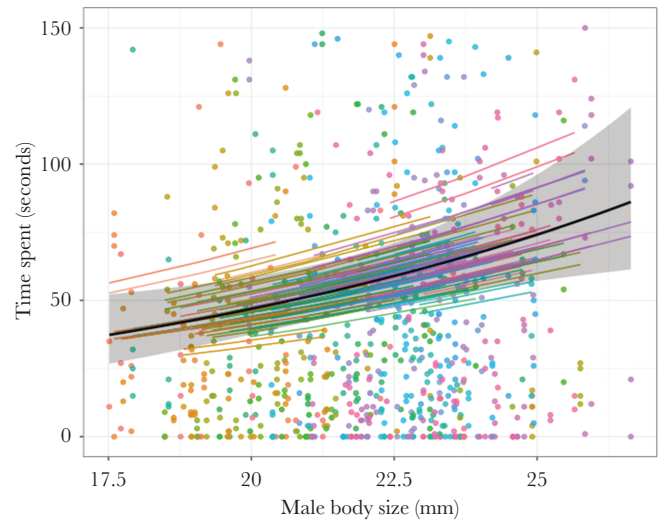
Figure 3
The relationship between male body size and time a female spent with a male. The black line indicates the among-block slope, with 95% confidence interval as a gray area, and the colored lines represent predicted slopes within each block (confidence interval not shown for clarity).

Table 3
Correlation coefficients, confidence intervals (in brackets) and *P* values (in italics) from multivariate linear mixed model testing the repeatability of female mate choice in eastern mosquitofish (*Gambusia holbrooki*) in first and second trials over two consecutive days. Bold values indicate significance at the 0.05 level

	Day 1 trial 1	Day 1 trial 2	Day 2 trial 1
Day 1 trial 2	**0.169 (0.030,0.285)** *0.022*		
Day 2 trial 1	**0.209 (0.090,0.335)** *0.002*	0.025 (−0.08,0.173) *0.404*	
Day 2 trial 2	0.021 (−0.141,0.161) *0.916*	**0.553 (0.422,0.639)** *<0.001*	**0.194 (0.034,0.322)** *0.016*

G. holbrooki. Contrary to findings in other species (review: Johnson and Gemmell 2012), we did not find an effect of male age on female mate choice in *Gambusia*. It should be noted, however, that most studies are observational and cannot distinguish between the effects of mating history and male age as the two traits tend to be highly correlated. Even so, our result is partly consistent with the findings of a study on another poecilid fish, the guppy *Poecilia reticulata*, where male age itself (without controlling for mating history) did not affect female mate choice (Gasparini et al. 2010). We also found no significant effect of male mating history on female mate choice. This was more surprising because a previous study of Poeciliids reported that females can distinguish between, and prefer, virgins over males that have mated (Scarponi et al. 2015). However, in that particular case, the study design exposed females to direct cues of male mating activity (i.e., females watched males mate), whereas we tested whether females could distinguish between males with different past mating history in the absence of any direct evidence as to how often males had mated, which better reflects the situation in the wild when a female encounters a male.

One possible explanation for our finding that female mosquitofish show no mate preference based on either age or experience is that they pay greater attention to conspicuous male traits, such as body size and gonopodium length (e.g., Kahn et al. 2010, 2012). Without experimentally eliminating variation in all other potentially preferred traits, it might, therefore, be impossible to detect small effects of male age or mating history. In the current study, for example, male body size had a large effect on female choice (see below). The ability of females to assess variation among potential mates is probably easier for stable phenotypic traits than subtle effects of age or past mating activity that alter their phenotype, and the resultant higher costs of accurate assessment might mitigate against any benefits that accrue from choosing older (or younger) males as mates. This is especially likely given that females mate multiply, which reduces the benefits of mate choice relative to those that arise through postcopulatory biasing of paternity (Radwan 2003; Fox et al. 2019). One concern that then arises is whether some aspect of our study design meant that females were simply uninterested in choosing a mate. This concern is, however, unfounded due to the observed strong female preference for larger males and the repeatability of female choice: females did not randomly associate with test males.

Male body size

Our finding that female mosquitofish exhibited a strong preference for larger males is consistent with earlier studies (e.g., McPeek 1992; Bisazza et al. 2001; Kahn et al. 2010, 2012; but see Bisazza and Marin 1991, 1995; Booksmythe et al. 2016). We further showed that there is an effect of both a male's relative size (i.e., females spent more time with the larger of four males within a block) and an effect of absolute male size (i.e., females spent more time with males in blocks where the mean male size was greater). This among-block effect could either be because females have a stronger preference for larger males the greater the absolute difference in size between the largest male and other available mates or simply because females prefer to spend time with any male if he is large. Several studies suggest that female *G. holbrooki* gain from associating with larger males because they make fewer mating attempts and, thereby, reduce the costs of sexual harassment (e.g., Hughes 1985; Bisazza and Marin 1991; Pilastro et al. 1997). There is, however, no evidence of a greater cost to females of being housed with a small than large male (Iglesias Carrasco, Fox, Vega-Trejo et al. 2019). Another benefit of associating with larger males is that they are socially dominant (Bisazza and Marin 1991, Harrison et al. 2018) so that, at low population densities, they can better repel rival males, which would, again, reduce the amount of sexual harassment (Bisazza and Marin 1991). Regardless of any benefits, our results suggest that a strong female preference for larger males might have overwhelmed our ability to detect a weaker preference based on male age or mating history. Nonetheless, our ability to document a preference for large males shows that our experimental design was sufficiently robust to detect female choice.

Repeatability of female choice

We found that female choice was significantly repeatable across trials within a day, indicating that the relative time a female spent with each male showed some consistency between trials (which was related to a male's size, see above). This result is similar to that in previous studies reporting repeatable female mating preference in guppies (Godin et al. 2005). In addition, we found that, despite a change in female mating status from virgin to nonvirgin between days, there was still repeatability of female choice across days. Similar evidence for repeatability that is independent of a female's social experience has been reported in killifish (Passos et al. 2013). Intriguingly, however, there also appeared to be a temporal effect in our study as there was a higher repeatability across days for the first or second trial, respectively, than there was between the first and second trial within a day. This suggests some sort of daily "habituation," which led to a decline in the strength of preference for a given male that was then "reset" between days. Further study is, however, required to better characterize this temporal variation in the repeatability of mate choice.

Female mating status

Multiple studies have highlighted that a female's mating status can affect her mate choice (e.g., Pitcher et al. 2003; Mellan et al. 2014; review: Kelly 2018). More specifically, a recent study on mosquitofish reported that nonvirgin and virgin females differed in their preference for computer animated males (Sommer-Trembo et al. 2017). In contrast, our study did not detect an effect of female mating status on mate choice: we found repeatability of female choice for specific males, which appeared to be due to a general preference for larger males. We did, however, find that nonvirgin females spent less time than virgin females associating with males, suggesting that they were less motivated to mate (see Bisazza et al. 2001; Mellan et al. 2014).

Future directions

Previous studies on *G. holbrooki* have shown that male age has complex interactions with his mating history that affect several sperm traits (Vega-Trejo et al. 2019). These traits could potentially affect a female's reproductive success. While male mating history itself does not affect male mating success (Iglesias-Carrasco, Fox, et al. 2019), it appears to have sex-specific effects on offspring life-history traits that could favor female choice for indirect, genetic benefits (Aich et al. 2020). Despite these potential reasons for females to discriminate based upon male age or mating history, we found that they had no detectable effect on female mate choice in our experimental study. We did, however, find that females prefer larger males, which is consistent with several previous studies (see above). Future research on the effects of male age and mating history on male mating success and, ultimately, their share of paternity would be useful to test whether females could indeed benefit by choosing males based on their age or mating history. We also recommend that future studies of *G. holbrooki* account for the effects of absolute and relative body size when testing if other traits affect female mate choice.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

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