

Title: Disentangling the effects of male age and mating history: contrasting effects of mating history on pre-copulatory mating behaviour and paternity success

**Running head:** Male age effect on reproductive success

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**Data accessibility:** All relevant data, and the code for calculating effect size from model are available at OSF: <https://osf.io/y9vb3/>, and also in Dryad: <https://doi.org/10.5061/dryad.wpzgmsbnp>

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## ABSTRACT

Many studies ask whether young or older males are better at acquiring mates. Even so, how age affects reproductive success is still poorly understood because male age and mating history are confounded in most studies: older males usually have more mating experience. To what extent does mating history rather than age explain variation in male mating success? And how do mating history and male age determine paternity when there is also post-copulatory sexual selection? Here we experimentally manipulated the mating history of old and young males in the eastern mosquitofish (*Gambusia holbrooki*). We then recorded male mating behaviour and share of paternity (1259 offspring from 232 potential sires) when they competed for mates and fertilizations. Old males, and males with no mating experience, spent significantly more time approaching females, and attempting to mate, than did young males and those with greater mating experience. Male age and mating history interacted to affect paternity: old males benefited from having previous mating experience, but young males did not. Our results highlight that the age-related changes in male

reproductive traits and in paternity that have been described in many taxa, may be partly attributable to male mating history and not simply to age itself.

## INTRODUCTION

Residual reproductive value and the likelihood of reproduction usually decreases with age because of a larger risk of dying due to somatic senescence (Williams 1966; Pianka and Parker 1975; Duffield et al. 2017). This leads to the general prediction that older males should invest more into their current reproductive effort, including effort expended on fighting for access to females and the intensity of their courtship (Pianka and Parker 1975; Kokko 1997; Brooks and Kemp 2001). In support of this claim, some studies show that older males expend more effort when fighting for mates (e.g., Kemp 2006; Fischer et al. 2008; Okada et al. 2020). Likewise, older males are often more persistent during courtship (e.g., Pérez-Staples et al. 2010; Prathibha et al. 2011; Karl and Fischer 2013; Rodríguez-Muñoz et al. 2019). Intriguingly, however, a new meta-analysis reported a non-significant trend for older males to invest *less* into sexual signalling (Dougherty 2021). This could reflect a counterbalance between an age-related decline in residual reproductive value, favouring greater mating effort, and older males having fewer resources available to invest in energetically costly sexual signals. It is therefore difficult to predict how male reproductive effort, and by extension mating success and paternity, will change with age (Brooks and Kemp 2001).

Regardless of any changes in mating effort, old males might have higher mating success simply because females prefer them as mates (Manning 1985; Proulx et al. 2002). This might occur because older males provide resources that increase female fecundity or offspring survival (e.g., Johnson et al. 2018; Prathibha and Jayaramu 2020). More generally, male longevity could act as an indicator of genetic benefits if offspring inherit paternal genes that increase their fitness by lowering the rate of mortality (e.g., Johnson et al. 2018). Any of these benefits could result in female choice biasing mating success towards older males, even if these males do not court more intensely (Kokko and Lindstrom 1996; Kokko 1997; also see Kokko 1998; Brooks and Kemp 2001; Kokko et al. 2002 for more inclusive discussion). However, females might also discriminate against older males if mating with them lowers fecundity or reduces offspring's viability (e.g., Jones and Elgar 2004; Pérez-Staples et al. 2010; Johnson and Gemmell 2012). Female choice could thereby reduce the mating success of

older males (e.g., Wedell and Ritchie 2004; McDonald et al. 2017; Lai et al. 2020). Furthermore, even if older males are more attractive, they might still have lower mating success. First, older males sometimes have a higher rate of failed mating attempts (Fricke and Maklakov 2007; Rodríguez-Muñoz et al. 2019; Okada et al. 2020). Second, in some species older males are more likely to lose fights, which reduces their access to females (Dean et al. 2010; Ruhmann et al. 2018; Lai et al. 2020).

Contrasting predictions for the effects of age on the determinants of mating success creates uncertainty as to whether pre-copulatory sexual selection favours older males. In some studies, older males have higher mating success (e.g., De Luca and Cocroft 2008; Fischer et al. 2008; Pérez-Staples et al. 2010; Somashekar and Krishna 2011), in others lower success (e.g., Dean et al. 2010; Kanuga et al. 2011; Johnson et al. 2018; Rodríguez-Muñoz et al. 2019), and in yet other studies there is no relationship between male age and mating success (e.g., Sawadogo et al. 2013). It is, however, noteworthy that these studies are almost all correlational. This raises questions about confounding factors that are correlated with male age that might produce an incorrect estimate of the direct causal effect of male age on mating success. One nearly universal confounding factor is a male's past mating experience, or reproductive effort. After all, old males are likely to have mated more often, or made a greater lifetime mating effort, than younger males (Vega-Trejo et al. 2019). It is well established that mating effort imposes physiological costs that lower body condition, suppress immune responses (Lawniczak et al. 2007; Bleu et al. 2016; Simmons et al. 2017), and ultimately increase mortality (Paukku and Kotiaho 2005). Higher past mating effort may therefore directly reduce a male's current mating success by making him less competitive (e.g., Koppik et al. 2018). However, several studies have shown that males can increase the success of their future mating attempts by gaining experience interacting with females (e.g., Dukas 2005; Dukas et al. 2006; Pérez-Staples et al. 2010; Milonas et al. 2011; Yasuda et al. 2015; but see Iglesias-Carrasco et al. 2019a; Thonhauser et al. 2019).

Although male age might affect mating success due to pre-copulatory sexual selection, fitness ultimately depends on how mating translates into fertilization, especially in the presence of post-copulatory sexual selection. The effect of age on reproductive success therefore depends on how age affects both pre- and post-copulatory success under sexual selection (Gasparini et al. 2019; Vuarin et al. 2019). In general, older males are less fertile (Fricke and Maklakov 2007; Hoikkala et al. 2008; García-Palomares et al. 2009; Kanuga et al. 2011; Sharma et al. 2015; Johnson et al. 2018; but

see Jones et al. 2007; Gasparini et al. 2010; Prathibha et al. 2011; Prathibha and Jayaramu 2020). As with studies correlating age with the expression of traits under pre-copulatory sexual selection, studies of fertility also tend to confound the effects of male age on ejaculates with those due to past mating effort. For example, the replenishment of sperm supplies following mating results in more germline cell division that potentially elevates mutation rates (Pizzari et al. 2008; Simmons et al. 2017). If haploid gene expression affects sperm traits (Immler 2019) increased mutation may lower male fertility. Therefore, past mating effort could reduce paternity under post-copulatory sexual selection, independent of male age.

To date, only a few studies on insects have experimentally disentangled the effects of male age and mating effort on mating success (Jones and Elgar 2004; Pérez-Staples et al. 2010; Wang et al. 2016; Koppik et al. 2018; Sepil et al. 2020). Their applicability to other taxa, especially vertebrates, is largely unknown (but see: Vega-Trejo et al. 2019; Aich et al. 2020). Likewise, very few studies have teased apart the effects of age and past mating effort on male reproductive success under post-copulatory sexual selection (insects: Jones and Elgar 2004; Radwan et al. 2005; Jones et al. 2007; Sepil et al. 2020; fish: Aich et al. 2021). Here, we experimentally manipulated the mating history of young and old male eastern mosquitofish (*Gambusia holbrooki*) so that mating history and age were uncorrelated. Using a full factorial experimental design, we tested how age and past mating experience affect mating behaviour, and paternity when males compete for both mates and fertilizations. Mosquitofish have internal fertilization and females usually have broods sired by multiple males (Zeng et al. 2017). Their mating system is characterised by coercive mating behaviour, with males rarely courting, and instead constantly chasing and attempting to copulate with females (McPeck 1992; Bisazza and Marin 1995). Males expend considerable time and energy attempting to mate, making up to one mating attempt per minute (Bisazza and Marin 1995; Wilson 2005). Past mating effort is therefore likely to affect a male's current mating behaviour (Chung et al. 2021; but see Iglesias-Carrasco et al. 2019a). In addition, to improve our ability to quantify the effects of male age and mating history on male reproductive success, we statistically accounted for other known sources of variation. Specifically, in mosquitofish, both male body size and gonopodium length (their intromittent organ) have been shown to affect male mating success and paternity (McPeck 1992; Head et al. 2017; Iglesias-Carrasco et al. 2019b; Kim et al. 2021), and higher heterozygosity has been linked to greater fertilization success (Head et al. 2017; Marsh et al. 2017; Vega-Trejo et al. 2017). By measuring male mating behaviour when males competed for access to females, and then quantifying each male's share of paternity, we could identify any discrepancy

between male mating success (inferred from our behavioural observations) and actual paternity. In our study the observed variation in paternity can be attributed to sexual selection and chance, as there was almost no natural selection. Fewer than <1% of males died while competing for mates and fertilizations.

## METHODS

### Study species: Origin and Maintenance

The eastern mosquitofish (*G. holbrooki*) is a poeciliid fish endemic to North America, but now found worldwide (Pyke 2005). This species was introduced to Australia in 1920's (Marsh et al. 2017). Mosquitofish have an average post-maturation lifespan of 12-15 months for females but far less for males. Some autumn-born females breed alongside their spring-born offspring the following year, but this seasonal overlap is absent for males (Pyke 2005, Kahn et al. 2013). In our field population it is unlikely that male mosquitofish live longer than 6 months. The time to maturity itself varies from 25 to 120 days in the laboratory (Vega-Trejo et al. 2016).

To determine how age and mating history affect male reproductive success in *G. holbrooki*, we bred 'young' and 'old' males and later randomly assigned them to one of two mating treatments (mated/naïve). We reared fish in single-sex 90L tanks (<50 individuals/tank) on a 14:10 h photoperiod at  $28 \pm 1^\circ\text{C}$ . We fed them *ad libitum* twice daily, with fish flakes in the morning, and *Artemia salina* nauplii in the afternoon.

To produce 'old' males, 400 adult stock fish (200 of each sex) were placed into 90L breeding tanks (<50 fish/tank) for 18 days to mate. The 200 females were then transferred to individual 1L tanks with a mesh barrier at one end to create a refuge for any fry produced. We checked the tanks twice daily for offspring. We then transferred up to 10 newborn fry per brood to 90L stock aquaria (< 50 fry/tank) over a 15-day period. We then repeated this procedure 12 weeks later with another 400

stock fish to produce 'young' males. All stock fish used to generate 'old' and 'young' males were collected in Canberra, Australia from September 2018-April 2019.

From four weeks after birth, offspring were inspected thrice weekly to determine their sex: immature males were transferred to male-only tanks to ensure the virginity of all individuals. Sexually mature males were identified by their fully-formed gonopodium with distal spines, and mature females by a visible gravid spot near their anal fin. When males reached sexual maturity, they were transferred to individual 1L tanks. Tanks were filled sequentially to allow matching of the time to maturity between 'young' and 'old' males later in the experiment. On average, 'old' males were 12-13 weeks older than matched 'young' males.

To disentangle male age from mating history, we manipulated whether or not a male could directly interact with, hence mate, a female. Each test male was housed in a 7L tank (17 × 28 x 15 cm) with a female. 'Naive' males had visual contact with a female, but a mesh barrier dividing the 7L tank prevented physical contact. 'Mated' males could interact with a female and mate freely (no barrier). The females were rotated among tanks every week to avoid a 'Coolidge effect' (see Vega-Trejo et al. 2014). These females were all wild-caught three months earlier, and held in female-only aquaria until used. We created 64 blocks of 4 males: one per age/mating treatment ('old/mated'; 'young/mated'; 'old/naive'; and 'young/naive';  $n = 64 \times 4$  treatments = 256 males). In each block, the males were randomly marked with one of seven different coloured elastomer tags for identification (following methods in Booksmythe et al. 2013). Although males resume their normal activity within 30–60 secs of tagging, we waited 24h before assigning them to mating treatments. After two weeks in their allocated mating treatment, males were removed for five days then stripped of their sperm (as part of a companion study) then returned to their mating treatment for another week. Further methodological details are in Aich et al. 2020.

### **Pre-copulatory male-mating behaviour**

At the end of the mating treatment, all males were kept alone for 5 days to replenish their sperm (O'Dea et al. 2014). We then ran mating trials, during which young and old males were

approximately 4 and 16 weeks post-maturity. This age difference is biologically relevant as it equates to around half of the maximum adult male lifespan in the wild population, and researchers have previously found a significant decline in sperm traits from weeks 3 to 14 after maturation (Vega-Trejo et al. 2019). In each trial a female was placed in a clear plastic cylinder in the centre of a 45cm x 45 cm tank filled with water to a depth of 10cm. The females were virgin daughters of wild-caught mothers. In each tank corner there was a compartment, into which we placed one male from each treatment. After a 5 min acclimation period, the female and four males were released to interact. We then observed the female for 40 minutes and recorded the behaviour of any approaching male. We recorded:

- i) the time spent by each male within two body lengths of the female.
- ii) the number of gonopodium swings within two body lengths of the female. Here the male moves his gonopodium laterally and then forward until it is almost parallel with his body.
- iii) the number of copulation attempts. Here the male swims quickly toward the female, swings his gonopodium forward and thrusts it at her gonopore (Garita-Alvarado et al. 2018).

In total, we ran 63 trials (N = 252 males from 63 of the 64 blocks; one block was unused after the death of a male). All trials were scored live by U.A., who was blind to male treatment type. The trials were also filmed, and the films are available upon request. Following these trials, males were then moved to the final experimental setup where they competed for paternity in a semi-natural setting.

### **Paternity success**

We then tested the ability of males to gain paternity when there is both pre-copulatory sexual selection (i.e., differential access to females) and post-copulatory sexual selection (i.e., sperm-competition and cryptic female choice). The four males from each of the 63 blocks used above were placed in a 90L tank with five virgin females. After 18 days, the males were removed and euthanised to collect tissue samples for DNA extraction. The females were transferred to individual 1L tanks each with a mesh barrier at one end to create a refuge for fry. The tanks were checked twice daily for offspring. Once a female gave birth, she and up to 10 randomly selected offspring were euthanised and tissue samples were collected. In total, 186 females from 58 blocks of males gave



birth to 1605 offspring (mean number of offspring per female = 8.63,  $s = 5.17$ ) of which 1316 were genotyped. Of those 186 females, 115 produced 10 or fewer fry; and we sampled 82% of the total number of fry born (1316 of 1605). DNA was collected from tail muscle/caudal fin of adults, and from the whole body (excluding the head) of fry. To assign paternity, we then genotyped single-nucleotide polymorphisms (SNP) for all the putative sires, females and offspring using a commercial service DArTseq (Kilian et al. 2012). This method has been successfully used to obtain paternity data in six previous studies on *G. holbrooki* (Bookmythe et al. 2016, Marsh et al. 2017, Vega-Trejo et al. 2017; Head et al. 2017; Spagopoulou et al. 2020, Aich et al. 2021). From these SNPs, a Hamming Distance Matrix for putative sires and offspring was calculated to determine paternity. As few as 30 optimized SNPs are sufficient to differentiate among 100,000 individuals based on Hamming Distance values (Hu et al. 2015): we used >4570 SNP loci. Each offspring was lined up against its four potential sires, and Hamming Distance values were compared. The sire with the lowest value was considered a match. We did not allocate offspring if their SNP distance values did not fall within two cut-off thresholds: i) if the percentage difference between the minimum and the next lowest distance was less than 10%, ii) if the absolute distance between an offspring and its most likely sire was greater than 0.25. Following these rules, we excluded 57 offspring, and ended up with 182 females from 58 blocks with 1259 offspring sired by 120 males. Inspection of Hamming distances did not reveal any sample mix-ups. Full methods for the paternity analysis are in the supplementary material.

Using the SNP data, we also calculated heterozygosity as the number of SNP loci that were scored as heterozygous divided by the total number successfully classified for each male ( $F_{\text{het}}$ : Head et al. 2017; Marsh et al. 2017; Vega-Trejo et al. 2017).  $F_{\text{het}}$  is essentially a measure of genome wide heterozygosity, and identical to  $1 - F_{\text{hom}}$ , as used by Bérénos et al. (2016). Paternity data were collected blind to male treatment.

### Statistical analysis

*Mating behaviour:* To assess how male age and mating experience affect mating behaviour we analysed (a) the time males spent associating with the female, (b) the number of gonopodium swings, and (c) the number of copulation attempts. In each case we ran generalised linear mixed

models (GLMM) with male age, mating history, their interaction, and whether or not the tag was yellow/red as a fixed factor. We included the interaction between mating history and tag colour as a fixed factor because tag colour had an unexpected, but significant, effect on male attractiveness in an earlier study of female choice (Aich et al. 2020). Tag colour might therefore have affected the mating rate of 'Mated males' during the mating treatment, hence their later mating behaviour and paternity success. We excluded the mating history by colour interaction from the final models as it was always non-significant for male mating behaviour (see **Supplementary material: Table S1**). We also excluded the age by mating history interaction from the model if it was non-significant to determine the main effects of age and mating history. We retained tag colour as a main effect in the final model as it might have affected female choice during the trials. We included block-centred male body size (i.e., male size – mean size in block) as a covariate to capture any effect of relative size. We treated Block ID as a random factor. In all cases, our data was right skewed and zero-inflated. To account for this, we used a gamma-hurdle model for the time spent associating with the female, and hurdle negative-binomial models for the number of gonopodium swings or copulation attempts. Hurdle models provide two separate fits: one for the probability of no response (i.e., presence/absence of the behaviour), and the other fits the non-zero responses.

We then re-ran the above models including block-centred male gonopodium size and then both block-centred male gonopodium size and male heterozygosity as covariates. These terms were not included in the initial models, as we first wanted to check for the main effect of male body size. For each of these models we were interested in whether the final term added explained any variation in male mating behaviour beyond that already explained. That is, for gonopodium size we were interested in variation explained after controlling for body size with which it is highly correlated ( $r > 0.90$ ). And for heterozygosity we were interested in variation beyond that attributable to morphological traits that might themselves be influenced by heterozygosity (Chapman et al. 2009). For this reason, we only report the test statistic for the added term. In all cases, inclusion of block-centred male gonopodium size and then both block-centred male gonopodium size and male heterozygosity as covariates did not affect the direction or significance of the main effects.

*Paternity:* To assess how age and mating treatment affect paternity, we used the number of offspring sired by each male with each female as the dependent variable in a GLMM. To account for over-dispersion and zero-inflation in our data, we assumed a hurdle negative-binomial distribution.

The model procedure was otherwise identical to that described for mating behaviours, with the exception that male ID, female ID and Block ID were treated as random factors as there was multiple occurrences of the males having offspring with different females in each block.

We then quantified three measures of repeatability corresponding to the two parts of the hurdle models and the full dataset. Repeatability was calculated as the Intraclass Correlation Coefficient (ICC). where:

$$ICC = \frac{\sigma^2_{\alpha}}{\sigma^2_{\alpha} + \sigma^2_{\varepsilon}}$$

Here  $\sigma^2_{\alpha}$  is among-individual variance and  $\sigma^2_{\varepsilon}$  is within-individual variance (Nakagawa and Schielzeth 2010). Repeatability was calculated using the function `qgicc` in the package `QggIimm` (De Villemereuil et al. 2016) to obtain each variance component. Further details of the calculations are provided in the supplementary material. We calculated the confidence interval of repeatability values by bootstrapping our model to calculate ICC for 1000 iterations, and used likelihood ratio tests to calculate p-values.

In all cases, the models were run using the R package `glmmTMB` (Brooks et al. 2017) in R Version 3.6.0 (R Core Team 2019). We set alpha = 0.05 and ran two-tailed tests. We always checked the distribution of residuals to ensure they met model assumption. Terms were tested for significance using the `Anova` function in the `car` package (Type III Wald chi-square tests).

To quantify the effect of male age, mating history and their interaction on variation in our three measures of male mating behaviour, and in paternity success we calculated the standardized mean difference (Hedges' *g*: Hedges 1981) between estimated values from our model predictions for: young and old males (male age effect); naïve and mated males (mating history effect); and the difference in values between young naïve and mated males versus old naïve and mated males (interaction effect). In all cases the model was run with truncated binomial family. We used the `predict` function to calculate the predicted means for each category of male from our model. We then simulated data from our model 1000 times, for each simulated data set we calculated the pooled standard deviation and took the mean pooled standard deviation across simulations. We then calculated 95% confidence intervals (CI) for each effect size using parameter re-sampling with a

multivariate normal approximation of the error variance-covariance: we drew parameter values from a multi-variate normal distribution of means corresponding to all parameter estimates and variance-covariance corresponding to the variance-covariance matrix of the parameters, and calculated the predicted mean response and effect size given this draw. We repeated the procedure 1000 times and took the 2.5% and 97.5% quantile of the distribution thus generating a 95% CI. Note that these predictions correspond to the full response variables (i.e., not divided into yes/no responses and conditional components given a non-zero response).

Finally, we conducted post-hoc pairwise comparison tests for the effects of mating treatment and age on paternity success. As our data was zero-inflated, we could not use the emmeans package (Lenth R. and Lenth M. R. 2018) in glmmTMB. We therefore fitted a similar model using the Bayesian R package MCMCglmm (Hadfield 2010) to conduct pairwise comparison tests. We ran a univariate GLMM with male age, mating history and their interaction, and block-centred body size as explanatory variables with a zero-inflated, over-dispersed Poisson model. Male ID, female ID and Block ID were treated as random factors. Using the full posterior distribution, we computed the predicted marginal mean response of all treatment combinations and then subtracted those predictions in a pairwise fashion to obtain credible intervals and Bayesian p-values for each of the six possible pairwise comparisons.

## RESULTS

### 1. Pre-copulatory male-male competition

There was no significant effect of male age, mating history, relative body size or heterozygosity on whether or not a male spent time close to the female (i.e., pursued her) (all  $p > 0.1$ ; **Table 1a**). However, males with a relatively shorter gonopodium were less likely to approach a female ( $\chi^2=10.443$ ,  $p=0.001$ ; **Table S3**). For those males that did spend time near a female, both male age and mating history had a significant effect on the amount of time (**Table 1a, Fig. 1a**). Old males and naïve males spent significantly more time near the female ( $\chi^2=6.773$ ,  $p=0.009$ ,  $\chi^2=7.602$ ,  $p=0.006$ ),

while male body size, relative gonopodium length and heterozygosity had no effect (all  $p > 0.1$ ; see **Table 1a** and **Supplementary Table S1-S4**).

Male mating history had a significant effect on whether or not males performed gonopodium swings: naïve males were more likely to do so ( $\chi^2_1 = 5.744$ ,  $p=0.017$ ; **Table 1b**, **Fig. 1b**). There was, however, no effect of male age on whether or not males performed gonopodium swings ( $\chi^2_1 = 0.52$ ,  $p=0.471$ ). Of those males that did perform gonopodial swings, neither their age nor mating history affected the number of swings ( $\chi^2_1 = 0.023$ ,  $p=0.881$  and  $\chi^2_1 = 1.151$ ,  $p=0.283$ ). Male body size, relative gonopodium length and heterozygosity also had no effect on whether they performed gonopodium swings or how many swings they performed (all  $p > 0.1$ ; **Table 1b** and **Supplementary Table S1-S4**).

Male mating history had a significant effect on whether or not males tried to copulate: naïve males were more likely to try ( $\chi^2_1 = 5.578$ ,  $p=0.018$ ). In contrast, male age did not significantly affect the likelihood of trying to copulate ( $\chi^2_1 = 1.173$ ,  $p=0.279$ ). Of those males that attempted to copulate, male age but not mating history had a significant effect on the number of attempts: old males made significantly more attempts ( $\chi^2_1 = 5.184$ ,  $p=0.023$ ; **Table 1c**, **Fig. 1c**). There was no effect of male body size, relative gonopodium length or heterozygosity on whether they performed copulation attempts or how many attempts were performed (all  $p > 0.1$ ; **Table 1c** and **Supplementary Table S1-S4**).

There was no significant interaction between male mating history and tag colour for any of the three measured male behaviours. There was also no interaction between male age and mating history for any of the three behaviours (all  $p > 0.1$ , see **Supplementary Table S1**).

## 2. Paternity success

There was no significant interaction between male age and mating history on whether or not a male sired offspring ( $\chi^2_1 = 0.092$ ,  $p=0.762$ ), but it did explain variation in the number of offspring sired if he did ( $\chi^2_1 = 4.559$ ,  $p=0.033$ ; **Table 2**, **Fig. 1d**). Post-hoc pairwise comparisons revealed that among

males that had previously had mating access to females, older males sired significantly more offspring than younger males (mean difference= 1.35,  $p=0.024$ ). No other pairwise comparisons were significant (all  $p > 0.1$ ; **Table 3**). Male body size, relative gonopodium length and heterozygosity all had no significant effect on whether or not a male sired offspring, or the number of offspring if he did (all  $p > 0.1$ ; see **Supplementary Table S1-S4**).

There was also no significant interaction between male mating history and whether the tag was yellow/red on whether a male sired offspring, or the number of offspring if he did (all  $p > 0.1$ ; see **Supplementary Table S1**).

### 3. Comparison of effect sizes

Although the effect sizes for each male mating behaviour and paternity success did not perfectly align (e.g., the interaction was significant for paternity but not for the mating behaviours), they also did not differ significantly from each other as can be seen by the high overlap in 95% confidence intervals (**Fig. 2, Table S5**). The only exception was that the effect size for male mating history was significantly larger for time spent with a female than it was for paternity (non-overlapping 95% CIs; **Fig. 2**). Naïve males spent significantly more time near females, but mating history did not affect paternity.

### 4. Repeatability

Finally, we tested for the repeatability of male reproductive success. The probability that a male gained some paternity across females was repeatable ( $R= 0.245$ ;  $CI= 0.147, 0.272$ ;  $p= <0.001$ ), but the repeatability of the number of offspring sired among males who gained paternity was zero ( $R= 0$ ;  $CI= 0, 0.105$ ;  $p=1$ ). Across all males, there was very low but significant overall repeatability in males gaining paternity and siring offspring ( $R= 0.004$ ,  $CI= 0.002, 0.159$ ,  $p= <0.001$ ).

## DISCUSSION

Studies testing for an effect of age on male reproductive success show no clear pattern across the range of taxa that have been investigated (reviewed in: Johnson and Gemmell 2012). This could be partly due to three key considerations that are often overlooked. First, most of these studies focus on traits under pre-copulatory sexual selection, such as those that influence female mate choice and male mating success, and fail to consider traits that affect male fertilization success, hence paternity (but see: Hoikkala et al. 2008; Gasparini et al. 2010; Pérez-Staples et al. 2010; Johnson et al. 2018; McDonald et al. 2017). Accurately testing for age effects on both male mating behaviour and paternity is, however, essential to quantify net sexual selection: success under precopulatory and postcopulatory competition can be positively or negatively correlated (Birkhead and Pizzari 2002; Simmons et al. 2017). Second, many studies investigating male reproductive senescence fail to test for effects of male age in a competitive scenario (e.g., they measure male fertility when a female only mates with a single male). But the outcome of male-male competition, be this via physical fights, competition for mates or sperm competition, is key to generating variation in male reproductive success. Inclusion of competition in an experimental design when testing for male age effects is therefore essential (Candolin 2000, Wong and Candolin 2005). Third, and most importantly, studies testing for male age effects rarely disentangle key factors that are likely to be correlated with age. In particular, male age and mating history are usually confounded: older males tend to have mated more than younger males. While there are a few studies that disentangle the effects of male age and mating history on male success under sexual selection in invertebrates (Jones and Elgar 2004; Pérez-Staples et al. 2010; Wang et al. 2016; Ekanayake et al. 2017; Koppik et al. 2018; Sepil et al. 2020), there are almost none in vertebrates (for exceptions see: Vega-Trejo et al. 2019; Aich et al. 2020).

Here, we experimentally separated the effects of male age and mating history on male mating behaviour and paternity success in the mosquitofish *Gambusia holbrooki* when males competed to mate with and then fertilize females. We could therefore determine whether age, mating history or their interaction affect male mating success, and their eventual share of paternity. We found that both older males and males with low past mating effort spent significantly more time chasing females and made more copulation attempts. We also found that males with low mating effort, independent of their age, made significantly more gonopodium swings toward females than males with higher past mating effort. These three male behaviours are all likely to be under pre-copulatory sexual selection, because they increase the likelihood of a successful mating leading to insemination.

This then raises the question of whether these behaviours predict reproductive success. We found that when young and old males with high or low mating effort competed for mating opportunities and then fertilizations there was a significant interaction between male age and mating effort on paternity. For males with higher mating effort, old males sired significantly more offspring than young males. In contrast, for males with low mating effort, there was no significant difference in the success of old and young males. We also found that the probability of males gaining some paternity was moderately repeatable ( $R = 0.25$ ).

Overall, our results support the claim that older males invest more than younger males into their current reproductive effort (meta-analysis: Dougherty 2021). The discrepancy in the significance of the effects of age and mating history on male mating behaviour and paternity suggests that pre- and post-copulatory sexual selection might act in different ways on age and mating history. Closer inspection of the effect sizes for age, mating history and their interaction (**Fig. 2**) shows, however, that despite our study having far larger sample sizes than is the norm in studies of poeciliids fishes, the low precision of the effect size estimates precludes conclusive answer.

### Male mating behaviour

Life history theory generally predicts that older males should increase their mating effort because residual reproductive value declines with age (Williams 1966; Parker 1974; Pianka and Parker 1975; Duffield et al. 2017). In agreement with this prediction, after controlling for mating history, we found that older male mosquitofish spent significantly more time chasing females and trying to copulate. Broadly similar findings have been reported in other taxa where older males are more persistent in the mating attempts and/or more successful at inseminating females (Jones et al. 2007; Fischer et al. 2008; Pérez-Staples et al. 2010; Somashekar and Krishna 2011; Karl and Fischer 2013; Ekanayake et al. 2017; Rodríguez-Muñoz et al. 2019; Okada et al. 2020; Šmejkal et al. 2021), although some studies also report that older males show a decline in courtship and mating attempts (Fricke and Maklakov 2007; Prokop et al. 2007; Dean et al. 2010; Kanuga et al. 2011; Ruhmann et al. 2018; Churchill et al. 2019; Lai et al. 2020). This general trend for old males to invest more in sexual signalling has been further supported by a recent meta-analysis (Dougherty 2021). The underlying problem when correlating age with male mating behaviour is that there are likely to be confounding effects of mating history that led to an overestimate of the role of male age itself. In our study, after controlling for male age, we found that males with high mating effort spent less time chasing



females, and made fewer gonopodium swings and copulation attempts. This could be because they have fewer resources available to invest into mating (review: Torres-Vila and Jennions 2005). This result is consistent with other studies where experimental elevation of mating effort leads to a subsequent decline in sexually selected male behaviours (e.g., King and Fischer 2010; Wang et al. 2016; Koppik et al. 2018; Macartney et al. 2020, but see: Hughes et al. 2000; Thonhauser et al. 2019; Iglesias-Carrasco et al. 2019a). Given the effect of mating history on sexual behaviour in *G. holbrooki* and the fact that age and mating history are tightly correlated, our finding bolsters our recommendation that it is necessary to control for male mating history when testing for age effects on sexually selected male traits.

### **Paternity success**

Male reproductive success depends on both mating success and how this translates into fertilizations under sperm competition and/or cryptic female choice (Birkhead and Pizzari 2002). The most valuable part of our study arguably lies in testing how male age and mating history affect paternity, and how well this is predicted by male mating success (or proxies thereof), which will depend on the extent to which pre- and post-copulatory sexual selection are aligned. For example, it has been suggested that there might be cryptic post-copulatory sexual selection against older males because of the potentially lower fitness of offspring sired by them due to a higher mutational load (Radwan 2003; e.g., bustards: Vuarin et al. 2019), which might negate any pre-copulatory advantage for older males (e.g., crickets: Rodríguez-Muñoz et al. 2019). We found that in *G. holbrooki*, unlike the case for mating behaviour where male age and mating history had independent effects, age and mating history interacted to determine paternity. For older males, more experienced individuals gained more paternity than naive males; but for young males the pattern tended to be reversed. One explanation for this is that older males with greater mating experience learn from their mating history and invest more in reproduction as a form of ‘terminal investment’ because their residual reproductive value declines with age. In contrast, younger males may not be as good at learning from their mating history as they have less motivation to invest extensively in reproduction early in their life (e.g., fruitflies: Dhole and Pfennig 2014). Our finding for older males aligns with studies showing that mating experience subsequently makes males more successful at courting or coercively mating with females (Dukas 2005; Dukas et al. 2006; Pérez-Staples et al. 2010; Milonas et al. 2011; Yasuda et al. 2015; but see: Iglesias-Carrasco et al. 2019a; Thonhauser et al. 2019; Sepil et al. 2020).

Our paternity results suggest that the effects of age and mating history on male mating behaviour do not completely predict reproductive success, hence that pre- and post-copulatory sexual selection on male age and mating history are imperfectly aligned. However, it is more appropriate to compare effect sizes to determine whether there is a significant difference *between* model estimates. In 8 out of 9 effect size comparisons for the three mating behaviours with paternity there is no significant difference. The one exception is that the effect of mating history on paternity and time spent with a female differ significantly from each other: naïve males spent significantly more time with females, while there was no effect of mating history on paternity. There is also low overlap between the 95% confidence intervals for the effect sizes for the interaction between age and mating history for paternity and time spent with a female. Thus, there is still evidence that pre- and post-copulatory sexual selection on male age and mating history differ. Any mismatch indicates that age and/or mating history have different effects on reproductive success under pre- and post-copulatory sexual selection.

It is important to note that ejaculate traits often play a major role in determining variation in male reproductive success (Gasparini et al. 2017; Koppik et al. 2018; Ruhmann et al. 2018; Gasparini et al. 2019; Vuarin et al. 2019). Although a previous study did not find significant effects of male age and mating history on female mate choice in *G. holbrooki* (Aich et al. 2020), cryptic female choice might modulate the effects of male mating success. More generally, male age and mating history could have different effects on mating behaviour and ejaculatory traits that affect sperm competitiveness. Ideally, we need a study with the same full factorial experimental design we have used here, but with artificial insemination of females with a mixture of sperm from old and young males with higher or past mating effort. This would allow us to quantify paternity under sperm competition, while removing variation due to mating success (see: Aich et al. 2021).

## CONCLUSION

Our results highlight the need to control for mating history when quantifying sexual selection on male age. Given a natural positive correlation between male age and mating history, previous correlational studies are unable to measure the direct effect of male age on sexually selected traits.

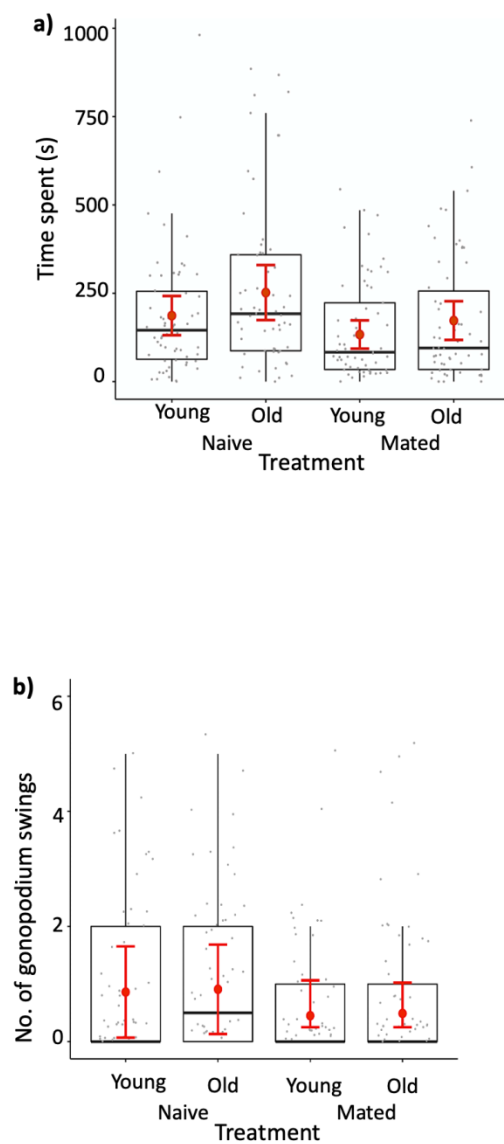
Using an experimental approach, we did so here for behavioural traits under pre-copulatory sexual selection in mosquitofish. We found independent effects of male age and mating history on mating behaviour. However, there was an interactive effect of male age and mating history on paternity. The difference between these two relationships suggests that age and mating history have different effects on pre-copulatory traits than they do on sperm competitiveness and/or cryptic female choice. This might reflect an age-mediated shift in the trade-off between investment in mating effort and ejaculatory traits, but this needs to be confirmed by direct quantification of the effects of age and past mating history on post-copulatory sexual selection.

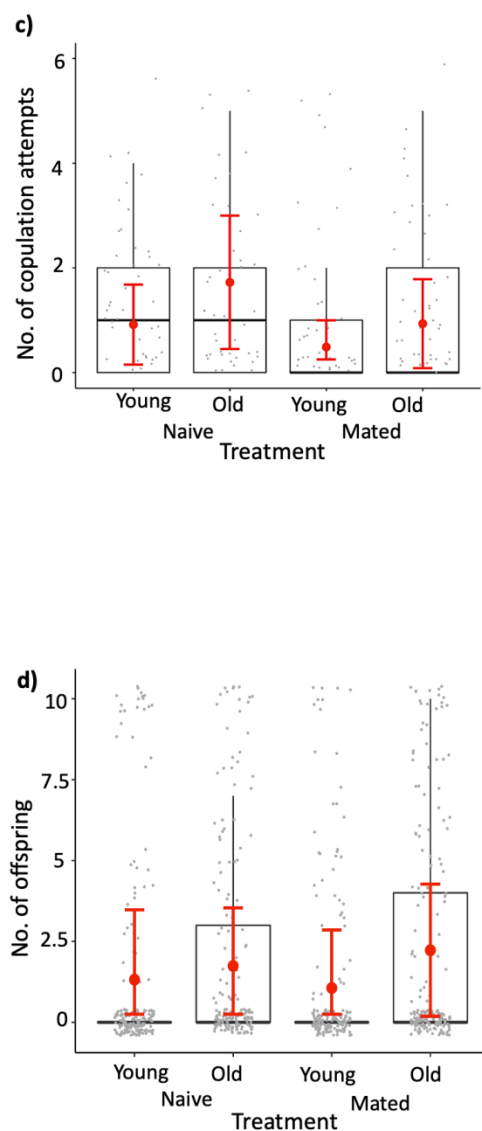
#### DATA ARCHIVING

All relevant data, and the code for calculating effect size from model are available at OSF: <https://osf.io/y9vb3/>, and also in Dryad: <https://doi.org/10.5061/dryad.wpzgmsbnp>

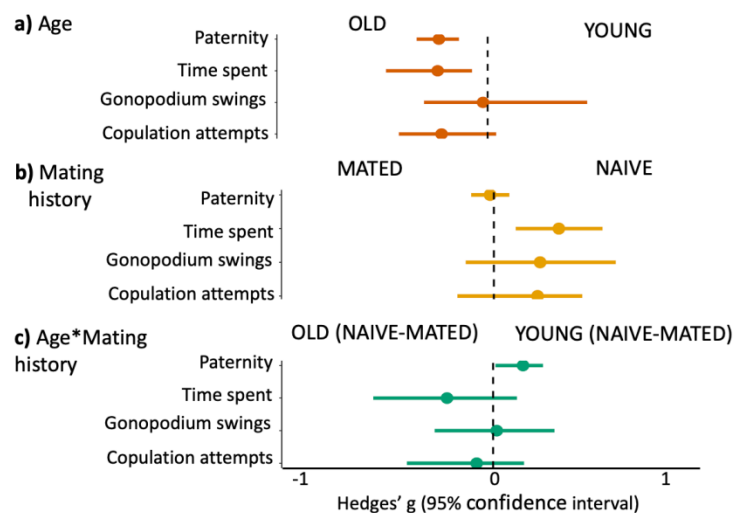
#### FIGURE LEGENDS

**Figure 1.** The effect of male age and mating history on: **(a-c)** pre-copulatory male-mating behaviour, and **d)** paternity success in *Gambusia holbrooki*. Box plots show median (horizontal line) and interquartile range of the raw data: **a)** time spent chasing female, **b)** the number of gonopodium swings, **c)** the number of copulation attempts ( $n=62 \text{ blocks} \times 4= 248 \text{ males}$ ); and **d)** the number of offspring sired ( $n= 1259 \text{ offspring from } 58 \text{ blocks of males}$ ). Mean and error bars in red represent the mean and 95% confidence interval values from the mixed model prediction.





**Figure 2.** Effect sizes (Hedges'  $g$ ) with 95% confidence intervals based on results from model predictions (see text) for **(a)** age, **(b)** mating history, **(c)** age\*mating history interaction for the four reproductive traits (paternity, time spent with female, gonopodium swings, copulation attempts). The black dashed line indicating no effect. The four effect sizes whose 95% CI do not overlap zero are significant at the 0.05 level. The effect sizes are presented as **(a)** Age: Young-Old, **(b)** Mating history: Naive-Mated, and **(c)** Age\*Mating history: (Young Naive - Young Mated) – (Old Naive - Old Mated).



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## TABLES

**Table 1.** Parameter estimates of zero-inflation and conditional models with Anova test statistics (type III Wald chi-square test) for the effect of male age and male mating history in eastern mosquitofish (*Gambusia holbrooki*) on their mating behaviour: **a.** Time spent near a female, **b.** Number of gonopodium swings, and **c.** Number of copulation attempts. The coefficients in the hurdle/ zero-inflation part of the model predict the probability of the behaviour not occurring, whereas the coefficients in the Gamma/ conditional parts predict the value conditional on the behaviour having occurred. Non-significant interactions were removed from the final model (see text and **Supplementary material** for full models). The bold font indicates significance at the 0.05 level.

	Predictor	Estimate	SE	$\chi^2$	P
<b>a. Time spent</b>					
	(Intercept)	10.486	2.686	15.240	<b>&lt;0.001</b>
<b>Binomial (Hurdle)</b>	Age (Young)	3.937	2.128	3.422	0.064

<b>Conditional (Gamma)</b>	Mating history (naive)	3.035	1.646	3.398	0.065
	Colour (Yes)	-2.257	1.707	1.748	0.186
	Relative body size	-0.467	0.734	0.405	0.525
	(Intercept)	5.184	0.133	1508.451	<b>&lt;0.001</b>
	Age (Young)	-0.317	0.122	6.773	<b>0.009</b>
	Mating history (naive)	0.329	0.119	7.602	<b>0.006</b>
	Colour (Yes)	0.136	0.123	1.223	0.269
	Relative body size	0.010	0.045	0.050	0.824

**b. Number of Gonopodium swings**

<b>Zero-inflation</b>	(Intercept)	0.849	0.313	7.372	<b>0.007</b>
	Age (Young)	0.211	0.292	0.520	0.471
	Mating history (naive)	-0.689	0.287	5.744	<b>0.017</b>
	Colour (Yes)	-0.246	0.285	0.748	0.387
	Relative body size	0.127	0.117	1.177	0.278
<b>Conditional (negative- binomial)</b>	(Intercept)	0.216	0.293	0.543	0.461
	Age (Young)	0.038	0.250	0.023	0.881
	Mating history (naive)	0.261	0.243	1.151	0.283
	Colour (Yes)	0.292	0.243	1.438	0.231
	Relative body size	0.120	0.090	1.792	0.181

## c. Number of Copulation attempts

Zero-inflation	(Intercept)	0.369	0.309	1.423	0.233
	Age (Young)	0.317	0.292	1.173	0.279
	Mating history (naive)	-0.680	0.288	5.578	<b>0.018</b>
	Colour (Yes)	-0.440	0.287	2.349	0.125
	Relative body size	0.022	0.115	0.038	0.846
Conditional (negative- binomial)	(Intercept)	0.717	0.261	7.554	<b>0.006</b>
	Age (Young)	-0.522	0.229	5.184	<b>0.023</b>
	Mating history (naive)	0.333	0.221	2.264	0.132
	Colour (Yes)	-0.022	0.221	0.010	0.920
	Relative body size	0.048	0.084	0.325	0.569

**Table 2.** Parameter estimates of zero-inflation and conditional models with Anova test statistics (type III Wald chi-square test) for the main and interactive effect of male age and male mating history on paternity success in eastern mosquitofish (*G. holbrooki*). The coefficients in the zero-inflation part of the model predict the probability of gaining no paternity, whereas those in the conditional part predict the number of offspring sired if a male gained some paternity. Non-significant interactions were removed from the final model (see text **Supplementary Table S1-S4** for full models). The bold font indicates significance at the 0.05 level.

Predictor	Estimate	SE	$\chi^2$	P
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	Predictor	Estimate	SE	$\chi^2$	P
Zero-inflation	(Intercept)	0.607	0.365	2.763	0.096
	Age (Young)	1.153	0.488	5.585	<b>0.018</b>
	Mating history (naive)	0.129	0.451	0.083	0.773
	Colour (Yes)	-0.212	0.331	0.409	0.522
	Relative body size	0.070	0.133	0.276	0.599
	Age (Young): Mating history (naive)	-0.200	0.662	0.092	0.762
Conditional (negative-binomial)	(Intercept)	1.701	0.096	313.832	<b>&lt;0.001</b>
	Age (Young)	-0.189	0.146	1.679	0.195
	Mating history (naive)	-0.171	0.134	1.615	0.203
	Colour (Yes)	0.013	0.040	0.858	0.354
	Relative body size	0.083	0.094	0.098	0.754
	Age (Young): Mating history (naive)	0.431	0.198	4.559	<b>0.033</b>

**Table 3.** Predicted marginal mean difference in the number of offspring sired and p values for pairwise comparisons of male age and mating history combinations in eastern mosquitofish (*G. holbrooki*). Naïve males = low mating effort; Mated males = higher mating effort. The bold font indicates significance at the 0.05 level.

Pairwise comparisons (parameter contrasts)	Mean difference	p-value
Old vs young males with higher mating effort	1.35	<b>0.024</b>
Old males with higher vs low mating effort	1.05	0.092
Old males with higher mating effort vs young males with low mating effort	0.955	0.160
Old vs young males with low mating effort	0.093	0.920
Young males with higher vs low mating effort	0.398	0.532
Old males with low mating effort vs young males with higher mating effort	0.305	0.524