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The effects of male age, sperm age and mating history on ejaculate senescence

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Abstract

1. In polyandrous species, a male's reproductive success depends on his ability to fertilize females, which, in turn, depends on his mating ability and his ability to produce competitive ejaculates. In many species, sperm traits differ between old and young males in ways that are likely to decrease the sperm competitiveness and fertility of older males. This age–ejaculate quality relationship is attributed to male ageing (i.e., senescence).
2. In a natural setting, male age and mating history are usually confounded: older males have usually mated and replenished their sperm supplies more often, so they have made a greater lifetime reproductive effort. In principle, the costs of reproduction, independent of any causal effect of male age, could generate an age-related decline in ejaculate quality.
3. To date, only a handful of studies have determined how male age, reproductive effort or their interaction affect ejaculate quality. Here, we experimentally manipulated the long-term mating history of 209 adult male mosquitofish (*Gambusia holbrooki*) over 14 weeks (N = 1,118 sperm samples). Males either had direct access to females and could mate freely, or had only visual and olfactory access to females. We documented the effect of mating history, adult male age (3, 9 and 14 weeks post-maturation) and their interaction on sperm velocity, sperm reserves and the rate of sperm replenishment. For sperm velocity, we additionally examined the effects of sperm age, because when older males mate less (or more) often than younger males there will be a correlation between mean sperm age and male age.
4. Sexually active males produced fewer sperm and replenished their sperm at a lower rate, and their sperm had lower velocity than males prevented from mating. Though older males produced more sperm, the rate of replenishment and velocity of their sperm was lower than the sperm of younger males. We also tested for a difference in the velocity of recently replenished (<24 hr) and older sperm (i.e., post-meiotic sperm senescence). There was no evidence that male age or mating history affects the extent of sperm senescence, but older sperm swam faster than recently produced sperm. Crucially, complex interactions are evident between

male age and male mating history with respect to sperm number and the proportion of sperm that are replenished.
5. These results suggest that male age and mating history will interact to determine the reproductive success of a male under sperm competition. They reveal a complex relationship between a male's age and his ejaculate quality. We suggest that both mating history and sperm age should be controlled for when measuring the intrinsic rate of senescence for male reproductive traits if the goal is to isolate effects that are solely attributable to a male's chronological age.

KEYWORDS
ageing, mating experience, mosquitofish, sexual selection, sperm competition, sperm quality

1 | INTRODUCTION

Determining whether reproductive success changes with male age has long interested evolutionary biologists (review: Lemaître & Gaillard, 2017). Theories of ageing/senescence predict that male performance will decline with age due to weakening selection, because extrinsic mortality reduces the proportion of a cohort that reach old age and are exposed to selection (review: da Silva, 2018). This could select for traits that enhance early life performance, even if they reduce late life performance (antagonistic pleiotropy: Medawar, 1952; review: Lemaître et al., 2015) or it could lead to the accumulation of mutations that are more often expressed in older individuals (Williams, 1957; see: Maklakov, Rowe, & Friberg, 2015; review: Brooks & Garratt, 2016). However, in some species, intermediate-aged or older males are preferred by females and/or are superior competitors when males fight for mates (Girndt, Chng, Burke, & Schroeder, 2018; Jones & Elgar, 2004). This has led to the controversial suggestion that older males, by virtue of having survived, signal that they will sire offspring of above average fitness, thereby making them more attractive to females (review: Brooks & Kemp, 2001). But pre-copulatory and post-copulatory sexual selection can favour different traits (Evans & Garcia-Gonzalez, 2016), so it is unclear whether a female preference for older males translates into greater male reproductive success, or higher female fitness. In particular, older males might produce ejaculates that are less successful under sperm competition (McDonald, Spurgin, Fairfield, Richardson, & Pizzari, 2017), or might be less fertile thereby lowering the reproductive output of monogamous females (Carazo, Molina-Vila, & Font, 2011; Dean et al., 2010). Furthermore, the accumulation of germline mutations might reduce the mean fitness of older males' offspring (Johnson & Gemmell, 2012; Johnson et al., 2018; Preston, Saint Jalme, Hingrat, Lacroix, & Sorci, 2015; Radwan, 2003a). Although the biological reasons why studies differ in the extent to which older males have higher or lower reproductive success, it is plausible that some of the variation is due to the extent to which older males accrue resources that increase their value as mates.

Recently, the effect of male age on traits under post-copulatory sexual selection, mainly "ejaculate quality" (sperm number, velocity, viability and motility), has received much attention ("ejaculate senescence": review: Pizzari, Dean, Pacey, Moore, & Bonsall, 2008). The relative number of sperm transferred is usually the key factor determining a male's sperm competitiveness and his share of paternity (reviews: Wedell, Gage, & Parker, 2002; Parker & Pizzari, 2010), and there is also weaker evidence that greater sperm motility, viability and velocity improve sperm competitiveness (Fitzpatrick & Lüpold, 2014; Simmons & Fitzpatrick, 2012; Snook, 2005). In humans, numerous studies report male age-related declines in ejaculate volume, sperm count, motility and viability (reviews: Kidd, Eskenazi, & Wyrobek, 2001; Johnson & Gemmell, 2012; Johnson et al., 2018). In other animals, sperm quantity declines with age in some species (Sasson, Johnson, & Brockmann, 2012), but increases in others (Gasparini, Marino, Boschetto, & Pilastro, 2010; Kanuga Manasi et al., 2010). Similarly, sperm velocity decreases with age (Gasparini, Marino et al., 2010; Moller et al., 2009), remains unchanged (Kanuga Manasi et al., 2010; Sasson et al., 2012) and even increases with age (Casselman & Montgomerie, 2004), depending on the species. There is also evidence from several species that older males have reduced sperm viability and a greater number of morphologically abnormal sperm (Stürup, Baer-Imhoof, Nash, Boomsma, & Baer, 2013).

Ejaculate senescence is likely to arise because older males have fewer resources (Wedell & Ritchie, 2004). Sperm production is costly, as demonstrated by the fact that males often strategically allocate sperm across matings (reviews: Wedell et al., 2002; Kelly & Jennions, 2011), and by the fact that sperm traits are plastically adjusted in response to social cues indicative of the likely level of sperm competition (Bretman, Gage, & Chapman, 2011). Given the costs of sperm production, any decline in general performance with age reduces the availability of resources that are needed to produce high-quality ejaculates. In addition, there is a trade-off between traits under pre- and post-copulatory sexual selection (model: Parker, Lessells, & Simmons, 2012; review: Simmons, Lüpold, & Fitzpatrick, 2017), and older males might invest less into ejaculates if the relative marginal gains decline with age (e.g., if older males are better at monopolizing females and thereby experience less sperm competition; Lüpold, Tomkins, Simmons, & Fitzpatrick, 2014). Age-dependent changes in resource allocation to ejaculates are likely to occur in most species

because the absolute acquisition of resources (i.e. condition; Rowe & Houle, 1996), which declines with age, usually affects optimal allocation decisions (see models by: Bonduriansky, Maklakov, Zajitschek, & Brooks, 2008; Tazzyman, Pizzari, Seymour, & Pomiankowski, 2009; Hooper, Lehtonen, Schwanz, & Bonduriansky, 2018).

Male mating history is a key factor that is often neglected, but should be taken into account, when investigating how male age affects ejaculate quality (Jones & Elgar, 2004). On average, older males have invested more heavily into courtship, sperm production and other costly traits associated with mating. Mating history could therefore partly explain age-dependent declines in ejaculate quality. The direct effects of male age and mating history need to be separated, either statistically or, ideally, experimentally. In addition, it is possible that there will be an interactive effect of male age and mating history. For example, male houbara bustards that initially invest more into extravagant sexual displays show a faster decline in the ability to invest in sperm production as they age (Preston, Jalme, Hingrat, Lacroix, & Sorci, 2011). A male's recent mating history can also affect ejaculate quality. When mating is frequent, ejaculate size partly depends on how quickly males can replenish their sperm supplies (O'Dea, Jennions, & Head, 2014). For example, in seed beetles the duration of the interval between copulations can be as important as male age in determining ejaculate size (Fricke & Maklakov, 2007).

Senescence can occur at both the organismal and the gametic levels: "post-meiotic sperm senescence" refers to the ageing of sperm cells, independent of male age (Pizzari et al., 2008; Reinhardt, 2007; Reinhardt, Dobler, & Abbott, 2015). This includes senescence of sperm stored by males before mating (Bressac, Damiens, & Chevrier, 2008), and post-ejaculation senescence of sperm stored by females (Kleven et al., 2009). Both processes occur despite the presence of specialized structures that function to keep sperm alive in both sexes. A key consequence of sperm senescence is that the number of viable sperm declines with sperm age. Post-meiotic sperm senescence is also apparent as a reduction in sperm motility and velocity (Gasparini, Kelley, & Evans, 2014; Gasparini, Daymond, & Evans, 2018; Pizzari et al., 2008; Vishwanath & Shannon, 1997; but see Firman, Young, Rowe, Duong, & Gasparini, 2015). Crucially, several recent innovative studies have shown that sperm competitiveness declines with sperm age (Gasparini et al., 2018; Gasparini, Dosselli, & Evans, 2017). These studies suggest that sperm age must be controlled for in age-based studies of male sperm traits.

Studies that investigate the effect of male age on ejaculate quality often have designs which limit the inferences that can be drawn. First, most studies are cross-sectional and compare the ejaculates of sets of different aged males. A limitation of this design is that genetic variation, parental effects and early life experiences that affect survivorship and ejaculate quality could hinder detection of age-dependent changes in ejaculates (Cornwallis, Dean, & Pizzari, 2014). Second, as already noted, many studies fail to control for a positive correlation between male age and mating history (but see Jones & Elgar, 2004 for an early attempt to experimentally tease them apart; see also Preston et al., 2011). Third, few studies take into account that when mating success changes with age, this could generate a correlation between male and sperm age (i.e., males that have not recently mated have older sperm).

To circumvent these limitations, we conducted a large, cohort-based, longitudinal study to evaluate how ejaculates change with male age in the eastern mosquitofish, *Gambusia holbrooki*. We experimentally manipulated male mating history by randomly assigning males to two treatments: either with constant access to females (mated); or separated from females by a mesh partition (unmated, but sexually primed). We controlled for sperm age by taking two ejaculate samples per male at each test age. The first sample comprised stored, hence older, sperm. The second sample comprised sperm that had been produced within the last 24 hr (i.e., after stripping sperm for the first sample). We investigated the effects of adult male age (3, 9 and 14 weeks post-maturation) and sperm age on: sperm velocity, reserves and production rate. In our field study population, most adult males seem to die within 16 weeks of maturation (Kahn, Kokko, & Jennions, 2013). Given that males are expected to invest continuously in reproduction, we predicted that sperm reserves, sperm production rate and sperm velocity would decline with both age (male senescence effect) and mating effort (cost of reproduction), and that these effects would interact such that old males with a history of mating repeatedly would have the lowest quality ejaculates. However, it is also possible that older males lacking mating opportunities could have higher quality ejaculates, if age-related declines in ejaculate quality that have been noted in previous studies are actually the result of increased prior mating effort. We also predicted that sperm velocity would decline with sperm age as a result of post-meiotic sperm senescence, and that this effect would be greater in older males and those with a history of mating repeatedly, with a possible interaction between male age and mating history.

2 | MATERIALS AND METHODS

2.1 | Study species

The eastern mosquitofish, *Gambusia holbrooki*, exhibits internal fertilization, with males transferring sperm to the female via a modified anal fin ("gonopodium"; Pyke, 2005). Males occasionally court, but mainly engage in coercive mating where they chase females and attempt to forcefully inseminate them (Bisazza & Marin, 1991). Males persistently attempt to copulate (up to one attempt/minute; Wilson, 2005). Females are polyandrous (Evans, 2011) and typically produce broods sired by multiple males (Booksmythe, Head, Keogh, & Jennions, 2016; Head, Kahn, Henshaw, Keogh, & Jennions, 2017; Vega-Trejo, Head, Keogh, & Jennions, 2017; Zane, Jones, & Avise., 1999). Sperm competition is therefore likely to be intense. Males can fully replenish their sperm reserves within five days (O'Dea et al., 2014). For species belonging to the family Poeciliidae, it is known that male reproductive success increases with the size of their sperm reserves and sperm velocity (e.g., green swordtails: Gasparini, Simmons, Beveridge, & Evans, 2010; guppies: Boschetto, Gasparini, & Pilastro, 2011). Additionally, there is evidence in poeciliids that males can accumulate sperm

for up to 60 days and do not dump or reabsorb sperm during storage (Billard & Puissant, 1969). However, little is known about how adult male age and mating history affect these critical sperm traits (but see Evans, Pierotti, & Pilastro, 2003) nor about how sperm age affects sperm performance in poeciliids.

2.2 | Origin and fish maintenance

We collected immature males and sexually mature females from two locations in Canberra, Australia (35°16′46.2″S 149°06′59.6″E and 35°14′30.1″S 149°06'17.0″E), during November and December 2017. We placed immature males in individual 1-L tanks where they were monitored daily to determine when they reached sexual maturity. We considered males to be mature when their gonopodium was translucent with a spine visible at the tip (Stearns, 1983; Zulian, Bisazza, & Marin, 1993). Females were housed in single-sex 90-L aquaria at densities of 0.33–0.67 fish/L. All fish were maintained under a 14:10-hr light: dark cycle at 28°C and fed ad libitum twice daily with *Artemia salina* nauplii and commercial fish flakes.

2.3 | Experimental design

To investigate the effects of adult male age (i.e., days since maturation), sperm age and male mating history on ejaculate traits, we conducted a longitudinal study in which we manipulated the mating history of males and measured their sperm traits at three ages (early, mid and late life). Upon reaching maturity (see above), males were randomly allocated to one of the two mating treatments. Males in the "mated" treatment (n = 103) were placed in a 7-L aquarium (17 × 28 × 15 cm) with a female whom they could pursue and mate. Males in the "unmated" treatment (n = 106) were placed in a 7-L aquarium which was divided in half with a mesh barrier (<2 mm weave). The male was placed on one side of the barrier and a female on the other side. The mesh barrier prevented the male from mating, but it allowed visual and olfactory cues from the female. Females were rotated weekly to maintain male interest in mating during the experiment.

2.4 | Experimental protocol

Adult males were stripped of their sperm (see details below) at 3, 9 and 14 weeks post-maturity. To do so, males were removed from their treatment tanks and placed in individual 1-L tanks 5 days before taking sperm measurements to allow full replenishment of their sperm reserves (O'Dea et al., 2014). After sperm were collected and analysed, males were then kept isolated for a further 24 hr, after which we again stripped them to measure both their sperm replenishment rate (i.e., sperm production/day; see Vega-Trejo, Jennions, & Head, 2016) and the velocity of sperm of a known age (24 hr). We compared the velocity of sperm from the first and second stripping to test whether older sperm are slower. After this second stripping, males were returned to their treatment tanks until the next set of measurements were made 5–6 weeks later.

2.5 | Sperm collection

To strip ejaculates, we followed the methods in Vega-Trejo et al. (2016). In brief, males were anaesthetized in iced water and placed on a glass slide under a dissecting microscope, the gonopodium was swung forward, and gentle pressure was applied to the abdomen to eject all available sperm in the spermiducts (Billard, 1986). Two samples containing three sperm bundles each were used for sperm velocity analyses, and the remaining bundles were collected with a pipette and transferred to an Eppendorf tube with extender medium (pH 7.5 with composition: 207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl2, 0.49 mM MgCl2, 0.41 mM MgSO4, 10 mM Tris (Cl)). The amount varied from 100 to 1,000 μl to ensure intermediate sperm concentrations that are required for accurate counts. Sperm collection and sperm measurements were all performed blind to male treatment.

2.6 | Sperm number

To estimate the number of sperm, we vortexed the sperm solution for 1 min and then mixed it repeatedly with a pipette (20–30 times) to break up sperm bundles and distribute the sperm evenly throughout the sample. We placed 3 μl of solution on a 20-micron capillary slide (Leja) and counted the sperm using a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) under 100× magnification. The threshold values defining cell detection were predetermined as elongation percentage 15–65 and head size 5–15 μm, and the static tail filter was set off. We counted five subsamples per sample and estimated count repeatability using the rptR package (Nakagawa & Schielzeth, 2010). Repeatability was high (r = 0.89 ± 0.005 *SE*, p < 0.001) so we used the mean of the five subsamples for further analyses. We corrected the total sperm counts by adding the average sperm number per bundle for the six bundles that we removed to estimate sperm velocity (Evans, 2009). The number of sperm per bundle does not vary significantly across individuals so we used the mean value (7,677 ± 477 *SE* sperm, n = 50 males).

2.7 | Sperm velocity

For each ejaculate, we used two samples (each with three sperm bundles and 2 μl of extender medium). We placed each sample in the centre of a cell of a 12-cell multi-test slide (MP Biomedicals, Aurora, OH, USA) previously coated with 1% polyvinyl alcohol solution (PVA) to prevent sperm from sticking to the slide. Each sample was then activated with a 3 μL solution of 125 mM KCl and 2 mg/ml bovine serum albumin (Billard & Cosson, 1992) and covered with a coverslip. We analysed sperm velocity within 30 s of activation for an average of 37.7 ± 0.6 *SE* sperm tracks per ejaculate (minimum 10 sperm tracks/male). We recorded two standard measures of sperm velocity: (a) average path velocity (VAP): the average velocity over a smoothed cell path and (b) curvilinear velocity (VCL): the actual velocity along the trajectory using a CEROS Sperm Tracker. The threshold values defining static cells were predetermined at 20 μm/s for VAP and 15 μm/s for VCL. Due

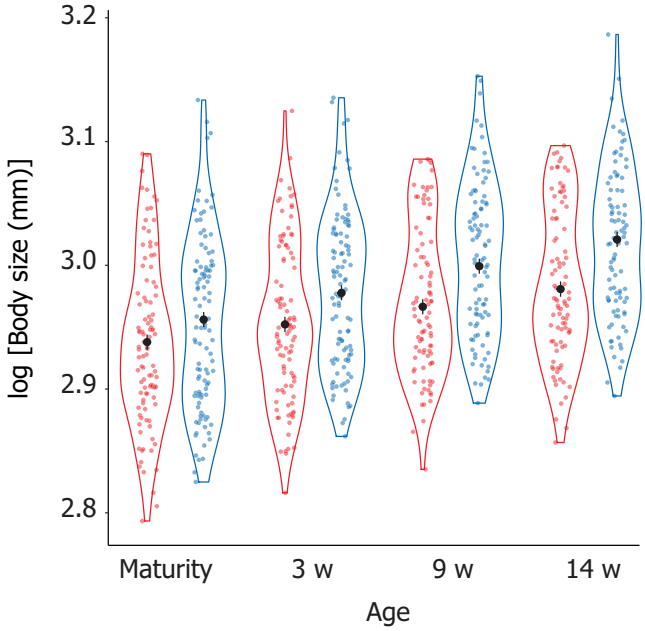

FIGURE 1 Post-maturation male growth. The effect of male age on body size (mm). Means ± *SE* from model predictions are shown in black. Violin plots represent the distribution of the data. Points represent raw data. Males from the mated treatment (maturity: $n = 103$, age 3w: $n = 103$, age 9w: $n = 93$, age 14w: $n = 91$) are shown in red, males from the unmated treatment (maturity: $n = 106$, age 3w: $n = 106$, age 9w: $n = 97$, age 14w: $n = 92$) are shown in blue

to the high correlation between VAP and VCL ($r = 0.97$, $p < 0.001$), we only used the more biologically relevant measure of VCL in our analyses (Boschetto et al., 2011). Given the significant repeatability of both parameters (VCL: $r = 0.53 \pm 0.024$, $p < 0.001$), we used the mean value in our analyses.

2.8 | Male morphology

All males were measured upon maturation and at 3, 9 and 14 weeks post-maturity. Males were anaesthetized by submersion in iced water for a few seconds to reduce movement and then placed on polystyrene with a microscopic ruler (0.1 mm gradation) and photographed. We measured male standard length (SL = snout tip to base of caudal fin) and gonopodium length using ImageJ software (Abramoff, Magelhaes, & Ram, 2004).

2.9 | Statistical analyses

2.9.1 | Male growth

To analyse male growth, we used a Generalized Linear Mixed Model (GLMM) to test whether male mating history influenced how body size changed over time. We used log male standard length (log-transformed) as our response variable and included the effects of mating history (fixed factor), age (continuous variable) and their interaction. We included male identity as a random factor in the model to control for repeated measures.

2.9.2 | Sperm number

To analyse the effect of mating history and adult age on sperm number, we used GLMMs. We ran separate models for each of our response variables: maximal sperm reserve (=initial sperm number from the first sperm collection at each age category), sperm production rate (=number of sperm replenished 24 hr after the first collection), proportion of maximal sperm reserve replenished (1 − [initial sperm number − number of sperm replenished]/initial sperm number). We square-root-transformed sperm number and the number of sperm replenished to fulfil model assumptions. In the case of the proportion of maximal sperm reserves replenished over 24 hr, we excluded 16 males from our analysis. Five were excluded due to the fact that they had no sperm at the initial stripping, and 11 outliers were excluded that displayed abnormally large sperm counts. Note that the inclusion of these outliers does not change our main conclusions.

In each model, we treated mating history (mated or unmated), male age (3, 9 or 14 weeks post-maturity) and their interaction as fixed categorical factors. We included log male standard length as a covariate, which was standardized and centred (mean = 0, *SD* = 1) within each age-class and mating treatment to aid interpretation (Schielzeth, 2010). We included the interaction with body size in all models.

2.9.3 | Sperm velocity

To analyse the effect of mating history and age on sperm velocity, we used a GLMM. We included mating history (mated or unmated) and sperm age (i.e., initial or replenished sperm) as fixed factors. We also included log male standard length as a covariate, but we did not include interactions with body size.

We treated researcher identity (as measurements were made by three individuals) and male identity as random factors in all models (sperm number and sperm velocity). All statistical analyses were performed using R version 3.2.4 (R Development Core Team, 2012), and the models were fitted using the lme4 package (v. 1.1-15). For all models with Gaussian error structure, we tested for an appropriate fit by checking the distribution of model residuals. We ran all models with and without interaction terms and compared the model fit of the reduced and full models using a log-likelihood ratio test. If removal of the interaction terms did not affect the model fit, we interpret the main effects from the reduced model. Finally, to test the significance of model terms we used the ANOVA function of the car package (v. 2.1-4), with type III Wald chi-square tests.

2.9.4 | Survival

To assess any potential effect of survival (live/dead) on the traits, we reran the models described above, but we included survival to 14 weeks as a fixed factor to see if it predicted variation in sperm traits from measures taken while males that did not survive were still alive. We ran separate tests for each trait at weeks 3 and 9 to

maximize the sample size for tests at each age. We therefore removed "age" and male identity from our models. We did not run tests for week 14 as there was no variation in survival.

3 | RESULTS

3.1 | Male growth

There was an effect of mating history on male growth. Male size increased with age, but at a slower rate for males who had mating access to females (Figure 1; interaction $p < 0.001$; regression of size on age for: mated males (estimate ± *SE*) = 2.938 ± 0.006; unmated males = 2.956 ± 0.006).

3.2 | Sperm traits

We collected sperm from 209 males (the number contributing to each age-class declined slightly as the experiment progressed due to male mortality). Male survival did not differ between the mated and unmated treatments ($GLM_{binomial\ error}$: $\chi^2 = 0.077$, $p = 0.781$). In total, 18 of 103 males from the mated treatment and 17 of 106 males from the unmated treatment died during the experimental period. In total, however, we still obtained sperm samples from 89.2% ($N = 1{,}118$) of the maximum of 1,254 samples (i.e., 209 males × 3 ages × 2 samples). Summary statistics are given in Table 1.

3.3 | Sperm number

3.3.1 | Maximal sperm reserves

At 3 weeks post-maturity, males from both mating treatments had similar numbers of sperm. However, while males from both treatments showed an increase in sperm numbers with age, males from the unmated treatment showed a steeper increase in their maximal sperm reserves than males from the mated treatment despite being given ample time (5 days) to replenish their full sperm reserves prior to being stripped (Table 2a, Figure 2a) at weeks 9 and 14 post-maturity. Bigger males also had larger sperm reserves than smaller males although the effect of male size was only just significant and was small ($r = 0.07$; Koricheva, Gurevitch, & Mengersen, 2013, $p = 0.045$, Table 2a).

3.3.2 | Sperm production rate

Males from the unmated treatment replenished sperm faster than males from the mated treatment (i.e., greater numbers of sperm 24 hr after the first collection; Table 2b, Figure 2b). We also found that across all age groups and treatments, bigger males replenished their sperm faster than smaller males. There was no detectable effect of male age on the number of sperm replenished (Table 2b).

3.3.3 | Proportion of maximal sperm reserve replenished

We found that both adult male age and mating history influenced the proportion of a male's sperm reserves that he replenished (Table 2c, Figure 2c). While both mated and unmated males showed an overall decline with age in the proportion of sperm reserves they replenished in a 24-hr period, at 3 weeks post-maturity males from the unmated treatment had replenished a higher proportion of their sperm than males from the mated treatment, while at 9 weeks post-maturity, the reverse was the case, with males from the mated treatment replenishing a higher proportion of their sperm reserves. The difference in replenishment rate between mating treatments was negligible at 14 weeks post-maturity. Male standard length had no effect on the proportion of sperm replenished.

3.4 | Sperm velocity

Sperm velocity decreased with adult male age in both treatments, and males from the mated treatment had slower sperm than those from the unmated treatment (Table 3, Figure 3). We also found that older sperm were faster swimming than recently produced sperm for all three male ages that we looked at (Table 3). There was no interaction between male age and mating treatment. Male standard length had no effect on sperm velocity (Table 3).

3.5 | Survival

There was no evidence that our results were biased by cohort heterogeneity. There was no effect of male survival on sperm number at week 3 ($\chi^2 = 0.007$, $p = 0.934$) or week 9 ($\chi^2 = 0.019$, $p = 0.890$), no effect of male survival on sperm replenishment at week 3 ($\chi^2 = 0.103$ $p = 0.748$) or week 9 ($\chi^2 = 0.543$, $p = 0.460$),

Sampling information	Sperm number	Sperm velocity (μm/s)
3 weeks	2,843,059 ± 2,275,604 (208)	90.812 ± 13.796 (204)
3 weeks (replenished)	1,479,252 ± 1,221,197 (196)	86.393 ± 15.787 (164)
9 weeks	4,225,188 ± 3,024,664 (191)	73.230 ± 14.296 (186)
9 weeks (replenished)	1,791,762 ± 1,391,391 (173)	68.219 ± 12.686 (160)
14 weeks	4,582,251 ± 2,787,563 (183)	66.697 ± 13.349 (180)
14 weeks (replenished)	1,754,500 ± 1,185,502 (172)	63.572 ± 13.742 (161)

TABLE 1 Mean ± *SD* (*N* of males) from raw data separated by both male and sperm age for sperm number and sperm velocity. Replenished refers to 24 hr after the first collection

TABLE 2 Results from linear mixed models with parameter estimates and chi-square (χ^2) tests for mating history (mated or unmated), age (3, 9 or 14 weeks post-maturity), male standard length and their interactions on sperm numbers

Trait	Predictor	χ^2	*p*	Variance
2a. Sperm number *n* = 579 males = 209	Intercept	676.965	<0.001	
	Mating history	0.055	0.815	
	Age	12.986	**0.002**	
	Male standard length	4.005	**0.045**	
	Mating history × Age	31.722	**<0.001**	
	Male identity			83,880
	Sampler identity			<0.001
2b. Sperm number replenished *n* = 539 males = 196	Intercept	96.630	<0.001	
	Mating history	8.932	**0.003**	
	Age	1.850	0.397	
	Male standard length	7.757	**0.005**	
	Male identity			58,850
	Sampler identity			12,422
2c. Proportion of sperm replenished *n* = 521 males = 196	Intercept	97.524	<0.001	
	Mating history	1.451	0.228	
	Age	12.477	**0.002**	
	Male standard length	1.333	0.248	
	Mating history × Age	8.680	**0.013**	
	Male identity			0.005
	Sampler identity			0.010

Note. *p*-values in bold indicate significant values. The full model (including interactions) and the parameter estimates are provided in Supporting information Tables S2–S4.

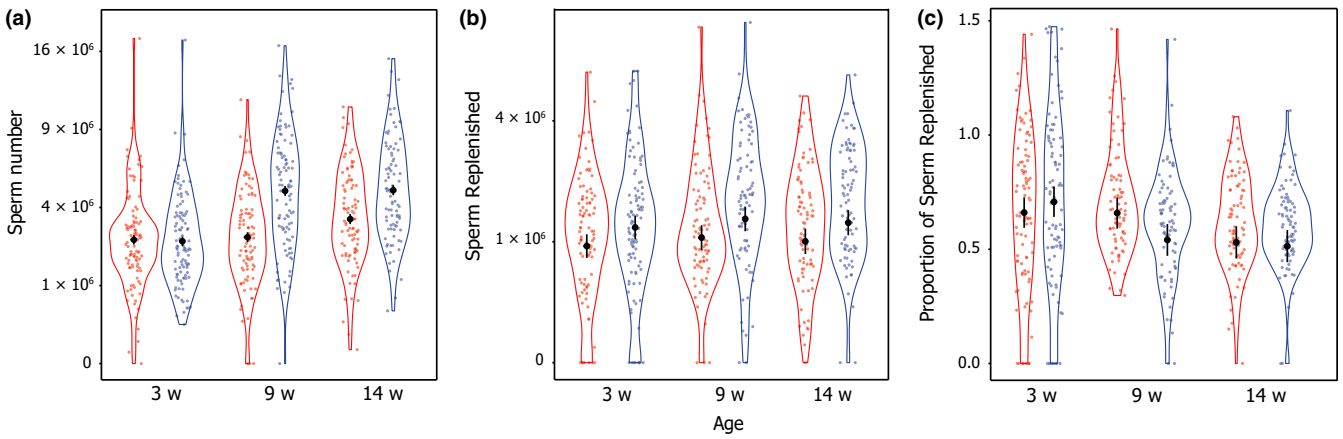

FIGURE 2 The effect of adult male age on (a) sperm number, (b) sperm replenished and (c) proportion of sperm replenished. Means ± *SE* from model predictions are shown in black. Violin plots represent the distribution of the data. Points represent raw data. In red are males from the mated treatment ([a] 3w: *n* = 103, 9w: *n* = 93, 14w: *n* = 93; [b] 3w: *n* = 96, 9w: *n* = 87, 14w:*n* = 85; [c] 3w: *n* = 93, 9w: *n* = 83, 14w:*n* = 84), and in blue are males from the unmated treatment ([a] 3w: *n* = 103, 9w: *n* = 98, 14w: *n* = 90; [b] 3w: *n* = 100, 9w: *n* = 86, 14w: *n* = 87; [c] 3w: *n* = 92, 9w: *n* = 83, 14w: *n* = 86). All ages are post-maturity

nor an effect of male survival on the proportion of sperm reserves replenished at week 3 (χ^2 = 0.692 *p* = 0.406) or week 9 (χ^2 = 0.123, *p* = 0.726). Finally, there was no effect of male survival on sperm velocity at week 3 (χ^2 = 0.065, *p* = 0.799) or week 9 (χ^2 = 0.173, *p* = 0.677).

4 | DISCUSSION

The idea that older males are more attractive to females because their survival indicates higher heritable fitness ("genetic quality") has proven difficult to test due to the many other factors that could

generate a relationship between a male's age and his value as a mate. For instance, sperm traits may change with age in ways that decrease sperm competitiveness and the fertility of older males (Pizzari et al., 2008; Siva-Jothy, 2000; Wedell et al., 2002). However, older males have usually courted, mated and replenished sperm supplies many more times than younger males (Reinhardt, 2007), so they have made a greater lifetime reproductive effort. Male age and mating history are therefore often confounded. Here, we examined the *independent* effects of adult male age and mating experience/effort on ejaculate quality in *Gambusia holbrooki* by experimentally controlling for male mating history. We found no negative effect of adult male age on the number of sperm in a male's sperm reserves, although the swimming speed of sperm did decline with his age. There was also no age-related decline in a male's ability to replenish his sperm: old and young males replenished the same amount of sperm in 24 hr. However, because the total size of sperm reserves increased with age, this meant that the proportion of the sperm reserve being replaced over 24 hr declined with age. We further found that the opportunity to mate reduced male somatic growth, as well as our measures of ejaculate quality, suggesting that there were high costs of reproduction for males in our study. Specifically, males who had had mating access to females grew more slowly, had a smaller increase in the size of their sperm reserves with age, took longer to replenish sperm after being stripped, and had slower swimming sperm than males who did not mate. Our results suggest that ageing and male mating history interact to determine the reproductive success of a male, and reveal a complex relationship between a male's age and his quality as a mate.

4.1 | Investment in sperm and adult age

Contrary to our initial prediction, the number of sperm in a male's sperm reserves did not decline with adult age, although sperm velocity did. This result, although surprising, is in line with other findings that older males produce greater quantities of sperm (Bressac et al., 2008; Evans, Pitcher, & Magurran, 2002; Gasparini, Marino et al., 2010). The finding of decreased sperm velocity with male age also confirms patterns recorded in other taxa such as barn swallows (Moller et al., 2009) and black-footed ferrets (Wolf et al., 2000). Producing both numerous and fast-swimming sperm is likely to be costly (Wedell et al., 2002), and our result suggests that older males might face a resource trade-off between sperm quantity and quality. The mechanism behind the potential trade-off could be explained in at least two ways. First, if the marginal costs of producing additional sperm are less than those of producing higher quality (fast-swimming) sperm in *G. holbrooki*, then it might be possible for young males to simultaneously invest in sperm quantity and quality when resources are abundant. However, as males age and available resources decrease due to, for instance, a decline in foraging efficiency or immune function, an age-related trade-off emerges that favours investment in sperm number over velocity, particularly if mating is costly (Wedell & Ritchie, 2004). Second, greater investment in sperm number over velocity by older males might represent the relatively higher benefits of more rather than faster sperm. Under sperm competition it is the number of sperm transferred, rather than sperm motility, which is usually key to determining a male's reproductive success (reviews: Wedell et al., 2002; Parker & Pizzari, 2010; but see Snook, 2005). This makes it likely that an old male will maintain his investment in sperm number, at the expense of sperm velocity. The fact that fitness outcomes associated with any quantity–quality trade-off depend on the social context could also explain why sperm quantity

TABLE 3 Results from a mixed model with parameter estimates and chi-square (χ^2) tests for mating history (mated or unmated), age (3, 9 or 14 weeks post-maturity), sperm age (initial = older sperm or replenished = ≤24 hr old sperm) and male standard length on sperm velocity

Predictor	χ^2	*p*	Variance
Intercept	343.008	<0.001	
Mating history	468.308	**<0.001**	
Age	8.821	**0.003**	
Male standard length	0.487	0.485	
Sperm age	27.643	**<0.001**	
Male identity			11.790
Sampler identity			61.270

Note. *p*-values in bold indicate significant values. The full model (including interactions) and the parameter estimates are provided in Supporting information Table S5.

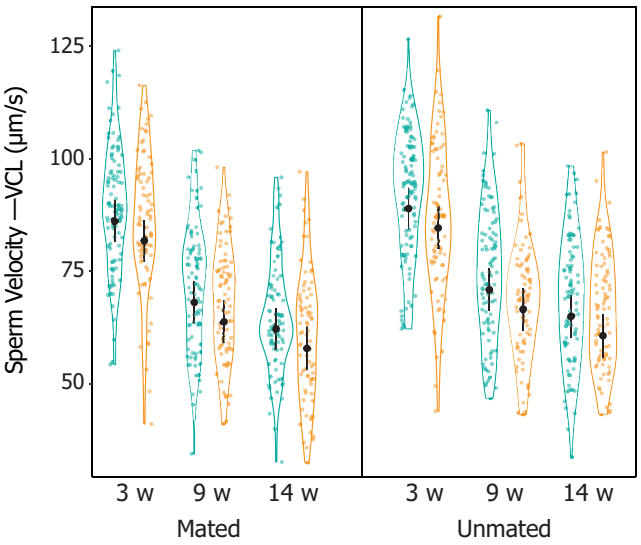

FIGURE 3 The effect of adult age on sperm velocity. Means ± *SE* from model predictions are shown in black. Violin plots represent the distribution of the data. Green indicates initially sampled sperm (>5 days old). Orange indicates replenished sperm (≤24 hr old). Points represent raw data. Mated initial sperm (3w: *n* = 99, 9w: *n* = 89, 14w: *n* = 90). Mated replenished (3w: *n* = 80, 9w: *n* = 81, 14w: *n* = 76). Unmated initial sperm (3w: *n* = 105, 9w: *n* = 97, 14w: *n* = 90). Unmated replenished (3w: *n* = 84, 9w: *n* = 79, 14w: *n* = 85). All ages are post-maturity

decreases with age in some taxa (e.g., in horseshoe crabs, where an older male is more likely to be "paired" and sperm competition is therefore lower for older males; Brockmann, Colson, & Potts, 1994; Sasson et al., 2012), and increases or stays constant with age in others (e.g., guppies: Gasparini, Marino et al., 2010; zebrafish: Kanuga Manasi et al., 2010; and mosquitofish: this study). Although we do not know the relationship between male age per se and the ability to monopolize females, we do know that larger males are better able to monopolize females (Bisazza & Marin, 1991) and given that males grow (albeit only a small amount) after maturing there might be a weakly correlated effect of male age on female monopoly.

4.2 | Investment in reproduction and sperm traits

It has been suggested that older males suffer sperm depletion (Wedell et al., 2002; Wedell & Ritchie, 2004), because the ability to replenish sperm decreases with age (Radwan & Bogacz, 2000). However, we found no evidence to support this premise in *G. holbrooki* because the rate at which males replenished sperm did not decline with age. This may reflect a mating system where males mate constantly, such that selection for sperm replenishment is consistently strong throughout a male's reproductive life (Gasparini, Marino et al., 2010). In contrast, however, we found that sperm replenishment in mosquitofish was negatively affected by a male's past reproductive effort. Other studies suggest a similar, strong effect of past reproductive effort. For example, in bushcrickets males who have mated more often have fewer sperm and lower nitrogen content in their spermatophore, even when given free access to food and time to recover between matings (Wedell & Ritchie, 2004). In general, high costs of past reproduction, independent of male age, could result in a decline in ejaculate quality with age. For example, in bulb mites, younger males are more successful under sperm competition, although it is unclear whether older males are less competitive because of their age per se or because they have mated more times (Radwan, Michalczyk, & Prokop, 2005). Producing sperm continuously necessarily increases the availability of young sperm (Reinhardt, 2007). Whether selection favours younger (or older) sperm because they are more competitive, or females preferentially retain younger (or older) sperm remains to be tested in mosquitofish. However, paternity is biased towards freshly inseminated sperm over that stored from a previous reproductive cycle in another poeciliid, the guppy (Gasparini et al., 2018), so the ability to replenish sperm and inseminate females with it will be strongly selected for.

By experimentally manipulating a male's mating history, we documented several costs of reproduction for male growth and ejaculate quality, while controlling for age as a confounding factor. Mating effort is associated with the allocation of energy resources to activities such as mate searching, courtship and copulation (Andersson, 1994), at the expense of investment in other traits. For example, increased sexual effort in the presence of females reduces post-maturation growth in guppies (Jordan & Brooks, 2010; Miller & Brooks, 2005). This is similar to our results, as males with mating access to females had a slower growth rate post-maturity than those that could interact, but not mate, with females. Greater reproductive effort could also explain why males in our study that had mating access to females had lower sperm reserves (even when given time to replenish) and slower swimming sperm than males that could not mate. Previous studies have demonstrated reductions in the number of sperm over consecutive matings in other species (Wedell et al., 2002). For example, larger male Soay sheep that mate frequently transfer fewer sperm per ejaculate over the mating season (Preston, Stevenson, Pemberton, & Wilson, 2001). Similarly, ejaculate size decreases over successive matings in several insect species (Alavi, Elgar, & Jones, 2016). Males in the mated treatment in our study presumably only allocated a small proportion of their sperm reserves with each consecutive mating, which might result in a less pronounced difference between our treatments than if males were to transfer their full sperm reserves each time they mated. It is also possible that males adjust sperm velocity according to the availability of potential mates (Bozynski & Liley, 2003; Gasparini, Peretti, & Pilastro, 2009), which might have been affected by males in the unmated treatment failing to gain access to females. It is, however, unclear whether these declines in ejaculate size are due to lower sperm production, or simply a short interval between matings. In contrast, other studies have shown that males respond to high mating rates by increasing the number and velocity of sperm, seemingly in anticipation of greater sperm competition (Devigili, Doldan-Martelli, & Pilastro, 2015), or by increasing their rate of courtship display and sneak copulation attempts (Miller & Brooks, 2005). This might partially explain why male *G. holbrooki* with high reproductive effort replenished sperm faster than those with a low reproductive effort, at least at an intermediate age (see Table 2b). More broadly, the observed effects of mating on ejaculate traits provide an example of the trade-off males face between allocation of resources into current and future reproductive effort (Reznick, Nunney, & Tessier, 2000). Our results add to evidence based on experimental manipulation of the constraints on ejaculate production when mating effort is high. More sexually experienced males essentially pay a price for their investment in current reproductive effort through reduced future sperm competitiveness (fewer and slower sperm). An interesting line of future research would be to test whether the greater growth in body size of males that avoid the costs of mating translates into a mating advantage, due to either female mate choice or to success during direct male–male competition for access to females favouring larger males.

Despite recent advances in incorporating sexual selection into life-history theory, the argument that indirect genetic benefits drive female mate choice for older males because they have shown their ability to survive remains controversial (Beck & Powell, 2000; Hansen & Price, 1995; Johnson & Gemmell, 2012; Kokko, 1998; Proulx, Day, & Rowe, 2002). The assumption that it is advantageous for females to mate with older males only works if all other things are equal (Trivers, 1972; highlighted by Brooks & Kemp, 2001). One assumption is that older males do not trade off lifespan against other fitness components, such as ejaculate quality. There is conflicting evidence about the reproductive success of

females mating with older males: some studies report negative consequences (Hale, Elgar, & Jones, 2008; Jones, Featherston, Paris, & Elgar, 2007), others no effect on reproductive success (Fricke & Maklakov, 2007), and still others greater fertilization success for females mated with intermediate-age males (Jones & Elgar, 2004; Jones et al., 2007). Declines in sperm function due to mutations in the germline (Hansen & Price, 1995; Radwan, 2003b) could also mean that fertilization by old males imposes indirect costs on females through the production of lower quality offspring (Gasparini, Marino et al., 2010; Johnson et al., 2018). Here, we addressed the issue of whether ejaculate quality that potentially influences a male's value as a mate is affected by age in *G. holbrooki*. We found unexpected differences in the ejaculate traits of old and young males: sperm reserves increased with adult age, while sperm velocity declined. Crucially, however, we found that a male's mating history has a *greater* influence on male ejaculate quality than his age. Studies that fail to control for mating history will therefore exaggerate the extent to which old and young males inherently differ. Our findings highlight the importance of potential interactions between male age and mating history that could influence sperm traits and, by extension, estimates of the effect of age on fitness (Johnson & Gemmell, 2012). Surviving to an old age might be an honest indicator of heritable variation in male viability, but it is ultimately the interaction with mating history that may prove key in determining the net fitness benefits for females of choosing older males.

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AUTHORS' CONTRIBUTION

M.L.H. and M.D.J. conceived the ideas of the project. M.L.H., R.V.-T., M.I.-C. and R.J.F. designed the methodology. R.V.-T., M.I.-C. and R.J.F. collected the data. R.V.-T. analysed the data. R.V.-T. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data are deposited in the Dryad Digital Repository https://doi.org/10.5061/dryad.8p502cr (Vega-Trejo, Fox, Iglesias-Carrasco, Head, & Jennions, 2019).

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