Disentangling the effects of male-male competition, reproductive investment and social dominance on telomere length

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Abstract

Energetically costly events can accelerate telomere loss and ageing via oxidative damage. For adult males, increased levels of reproduction and more frequent fighting for access to mates are therefore stressful experiences that should hasten telomere shortening. Testing for these relationships is, however, confounded by potential correlations between social status and reproductive investment. We used a 2 x 2 experimental design to test how long-term winning or losing contests, in combination with variation in reproductive effort, influenced telomere loss and several key life-history traits in the eastern mosquitofish, *Gambusia holbrooki*. After 9 weeks there were significant differences between winners and losers in their pre-copulatory reproductive investment (i.e., mating effort), but not in their post-copulatory reproductive investment (i.e., ejaculates). Males that were previously able to mate with females (i.e., had greater past reproductive investment) had significantly lower current mating effort, lower body growth, and slower swimming sperm, but only when males were small. These findings suggest that mating costs incurred from both consistent contest experiences and past reproductive effort depend on male body size. Finally, males that had previously been able to mate with a female did not have shorter telomeres than males that were unable to mate, while there was equivocal evidence that winners had shorter telomeres than losers. This intriguing finding hints at the important role social dynamics play in determining relative male investment into reproduction and somatic maintenance.

Teaser Text

For adult males, the costs associated with fighting for mating opportunities and then mating can accelerate telomere loss and ageing. This is because telomeres are susceptible to oxidative stress induced by elevated levels of sex or stress hormones. Mitigating the effects of oxidative stress is energetically expensive, so elevated investment into reproduction is assumed to trade-off with reduced investment into somatic maintenance. Testing for these relationships is, however, confounded by potential correlations between social status and reproductive investment. We experimentally manipulated the social status of male eastern mosquitofish via 'winner-loser effects' for 9 weeks. In addition, winners and losers had either full or no access to mating opportunities during this period. Surprisingly, past reproductive effort did not affect telomere length, but there was evidence of mating costs on other life-history traits. Instead, males that consistently won fights significantly increased their mating effort and appeared to have shorter telomeres than males that consistently lost fights. These unexpected results hint that stressful social experiences later in life, namely always winning or always losing fights against novel rivals, might play a greater role in telomeric attrition that is frequently attributed to elevated reproductive investment.

Introduction

Stressful, energetically costly events can accelerate ageing and hasten death (Blackburn et al. 2015). At a mechanistic level, ageing and death can partially be attributed to the rate of telomeric attrition – whereby telomeres, repeated sequences of noncoding DNA found on chromosomal ends, reach a critically short length (Blackburn 1984). Telomeres shorten whenever somatic cells divide (Haussmann and Marchetto 2010), hence telomere length is correlated with life expectancy and lifespan (Dantzer and Fletcher 2015). Stress can accelerate telomere loss when reactive oxygen species (e.g., free radicals; ROS), produced by metabolic processes and immune cells, exceeds the capacity of antioxidant defences to mitigate or repair damage (Houben et al. 2008; Monaghan et al. 2009). Consequently, rates of telomeric attrition are linked to the general levels of stress experienced by animals (Angelier et al. 2018). Studies investigating the role of stress on telomeric attrition tend to focus on early-life environments (e.g., Nettle et al., 2015; Marasco et al., 2019; Eastwood et al., 2022). However, the level of reproductive effort and intensity of sexual competition are likely to be highly relevant stressors for adults that could accelerate telomere shortening, hence the rate of ageing (Chatelain et al. 2019).

For males, mating effort and reproduction elevate the expression of sex (e.g., testosterone) and stress (e.g., cortisol) hormones that increase oxidative stress because elevated testosterone and corticosteroid expression suppresses antioxidant defences (Harshman and Zera 2007; Heidinger et al. 2021). There is some evidence that telomere length declines with greater male investment into reproduction. For example, in several bird species, males that invest heavily into parental care (Bauch et al. 2016), or that have brighter plumage colouration (Parolini et al. 2017; Taff and Freeman-Gallant 2017), have elevated reproductive success but shorter telomeres. Additionally, the different ways that males can invest into reproduction might also influence the rate of telomeric

attrition. For example, the red and yellow headed colour morphs of male painted dragons (*Ctenophorus pictus*) have different reproductive tactics which seems to affect their telomere length (Rollings et al. 2017). The red-headed colour morph that invests heavily into winning fights has shorter telomeres than the yellow-headed colour morph that invests more in sperm competition (Rollings et al. 2017). However, it is unclear whether a male's initial telomere length affects which morph it develops into, hence its reproductive strategy, or whether the different strategies themselves affect rates of telomere loss, to generate the observed differences in adult telomere length between the morphs. Taken together, these intriguing findings suggest that male-male competition, a major facet of male reproductive investment, can increase rates of ageing and telomere loss.

Males in species that face more intense competition for access to females (i.e., polygyny) tend to have relatively lower lifetime breeding success (Lukas and Clutton-Brock 2014), due to costs associated with mating competition that reduce mean male lifespan (Lemaître et al. 2020a). Males that monopolise access to females face more frequent challenges from rivals, resulting in an increased risk of injuries or associated energetic costs (Goymann and Wingfield 2004), or increased investment into sexual traits at the expense of somatic maintenance (e.g. male crickets; (Hunt et al. 2004). Social dominance can even lead to accelerated ageing in some mammals. For example, high-ranking male *Papio cynocephalus* baboons show epigenetic ageing effects beyond those attributable to their chronological age, suggesting that social dominance accelerates ageing (Anderson et al. 2021). Faster ageing in high-ranking males might be partly due to increased oxidative stress experienced during the mating season as high-ranking males monopolise mating opportunities and breed more often (e.g., *Mandrillus sphinx* mandrills; (Beaulieu et al. 2014). Increased levels of courtship, higher mating rates, more frequent fighting for access to mates, and, presumably, stress associated with social dominance interactions, should all hasten telomere shortening because of the

higher energetic and physical costs associated with reproduction (Monaghan et al. 2009). Determining causality is, however, challenging. Social dominance is often determined by factors like physical condition, body size or fighting ability, that might be correlated with telomere length due to early life experiences. To the best of our knowledge, the effects of male-male competition and reproduction on rates of telomere shortening have not yet been experimentally separated.

Intriguingly, prior wins or losses can have carry-over effects on contestants whereby winners are more likely to win subsequent fights, and *vice versa* for losers (winner-loser effects: (Hsu et al. 2006). Winning males often experience a brief elevation in testosterone levels, which can increase aggression (Carré et al. 2013). In contrast, losing males experience decreased expression of testosterone, or an elevation in stress related hormones (Earley et al. 2013). While the outcome of a single contest is unlikely to shorten telomeres, a consistent imbalance of cortisol and testosterone following a history of consistently either winning or losing contests could exacerbate oxidative damage and accelerate telomeric attrition (Casagrande and Hau 2019). Males that consistently win contests might adaptively increase their allocation of investment into reproductive rather than somatic traits to take advantage of their winning status (Harrison et al. 2023). We already know that prior contest experience can influence male reproduction. For instance, after only a single contest between size-matched male fruit flies, winners are more likely to mate with females while losers are more effective at sperm competition (Filice and Dukas 2019). Similarly, winning male mosquitofish spend more time than losers with females (Harrison et al. 2018); and after a consistent, long-term experience of winning or losing contests, winning male mosquitofish have better pre-copulatory outcomes than losers (Harrison et al. 2023). These differences in the reproductive investment of winners and losers are likely to trade-off with other life-history traits, such as somatic maintenance, and even lifespan, as males attempt to maximise their relative fitness (Kirkwood and Rose 1991). This leads to the prediction that telomere length, and by extension

male longevity, will be altered by the winner-loser effect.

Here, we conduct a 2 x 2 experiment with a poecilid fish to test whether long-term winning or losing contests, combined with either access or no access to mating opportunities, hence the likely investment into reproduction, subsequently results in: a) differential investment into various components of reproduction (i.e., traits under pre- and post-copulatory sexual selection), b) different life-history strategies (e.g., growth and mortality rates), and c) differential rates of telomere shortening. Specifically, we predict that: (i) winners will invest more heavily than losers into reproduction over somatic maintenance; (ii) increased reproductive effort due to greater past access to females will accelerate ageing and shorten telomeres; (iii) the effect of winning/losing on telomere shortening is less clear because, while losing is stressful, it may also reduce the mating rate, hence net costs of reproduction. Previous studies have found that stressful environments accelerate rates of telomere shortening, but it remains unknown how the experience of consistently winning or losing contests influences telomere shortening. This is partly because an experimental approach is required. It remains untested how the outcome of contests, *independent* of an individual's condition, phenotype or prior life experience (i.e., confounding factors that affect fight outcome but might also be correlated with telomere length), affect telomere shortening in adults. In general, we expected any differences between winners and losers to be magnified when males had previously had the opportunity to mate because this should increase their total past reproductive investment. We therefore expected to see significant interactions between contest experience and mating treatment.

Methods

This study was pre-registered at the Open Science Foundation (OSF; https://osf.io/saj46/).

Study species

We investigated how long-term winning or losing, in combination with variation in reproductive effort, influences telomere length and life-history strategies in male eastern mosquitofish, *Gambusia holbrooki*. This promiscuous poecilid species has been used previously as a model organism to study winner–loser effects on male reproduction (Harrison et al. 2018, 2023). Males engage in agonistic interactions to establish dominance hierarchies (Harrison et al. 2018, 2023), and otherwise spend most of their time pursuing and attempting to 'sneak' copulate with females (Bisazza and Marin 1991; McPeek 1992). Males invest heavily into both competition and reproduction, and they tend to survive for only one breeding season (Kahn et al. 2013). As such, male mosquitofish likely trade-off reproductive investment and lifespan. Based on earlier work (Harrison et al. 2023) we expected that consistently winning or losing contests, and body size, will combine to mediate trade-offs between reproduction and telomere length/lifespan.

Animal collection and maintenance

Mature adult mosquitofish (males: presence of hook-like tip to the gonopodia; females: presence of gravid spot) were wild-caught from Sullivan's Creek, Canberra, Australia in December-January 2021-22. We collected recently matured adults at the beginning of the breeding season to minimize age variation among focal males. Fish were taken to the Australian National University and housed in dedicated 90 L same-sex stock tanks (~50 individuals per tank) at 29 ± 1 °C under 14 L:10 D light regime and fed *ad libitum* commercial fish flakes twice daily. All experimental work was conducted under protocol A2021/04 (ANU Animal Ethics Committee).

193

194 *Creating winners and losers*

195 We used a fully factorial experimental design to test for winner-loser effects, effects of reproductive
196 investment opportunities, and their interaction, on telomere length and several life-history traits
197 (Figure 1). We randomly selected focal male mosquitofish (n = 176) and anaesthetized them briefly
198 in an ice slurry to measure their standard body length (SL) and to individually mark them with a
199 subcutaneous elastomer tag (NorthWest Marine Technology, Washington, USA) positioned below
200 the dorsal fin. Tagged males were then isolated in individual 1 L aquaria for 1 week to minimize
201 the influence of recent social interactions on contest outcomes (Kasumovic et al. 2010). We
202 selected males from across the natural population size range (focal male SL mean ± standard
203 deviation: 20.43 ± 1.54 mm, range: 17.31-23.82 mm; n = 142; Table 1) to disentangle the effects of
204 winning/losing and body size on reproductive investment and relative telomere length. While exact
205 male age was unknown, males were randomly assigned to the four treatments so that, on average,
206 telomere length was initially expected to be the same for winners and losers.

207

208 Following 1 week of isolation, focal males were randomly assigned to become winners or losers by
209 being paired with either a smaller or larger rival. This experimental approach controls for intrinsic
210 differences between males that might otherwise determine winners and losers in natural encounters
211 (Hsu et al. 2006). Winning-losing experiences took place in 6 L aquaria that contained gravel and
212 plastic plants and had black plastic on three sides to minimize outside disturbance. Focal and rival
213 males freely interacted for 1 week, after which a female was introduced to begin the reproductive
214 opportunity treatment for a further 8 weeks. For the reproduction treatment, males in half the
215 winner/loser aquaria could freely interact with the female and fully invest in reproduction (i.e.,
216 mate, ejaculate and then replenish sperm); the remaining half had a female present behind a mesh
217 barrier to prevent mating from occurring (Figure 1). Rival males and stimulus females were rotated

every few days so that focal males continued to fight to establish dominance, and to pursue females (Vega-Trejo et al. 2014).

Pre-copulatory reproductive investment

After 9 weeks (approximately half the duration of the natural breeding season), each focal male was placed in a new 6 L aquaria with a random stock female to quantify his mating behaviour. All females were only used once. Male mating behaviour was observed for 20 mins where we recorded: a) time spent associating with the female (<5 cm from female and interacting with her), b) the number of mating attempts, and c) the number of successful attempts (i.e., had the potential to transfer sperm to the female). Once mating behaviour trials had ended, the female was removed and her body length measured using digital calipers (female SL mean ± standard deviation: 30.29 ± 3.34 mm, n = 142).

Post-copulatory reproductive investment

Immediately following mating behaviour trials, focal males were anaesthetised in ice slurry to measure their final body length and to strip their sperm (O'Dea et al. 2014; Vega-Trejo et al. 2016). Males were then isolated for 5 days in 1 L aquaria to replenish sperm reserves (O'Dea et al. 2014), after which we again stripped their sperm. We took two samples, each of three sperm bundles, from each male for sperm velocity analysis. The remaining bundles were collected into an Eppendorf tube with a known volume (range: 100-1200 μL) of 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)) for sperm counts. Sperm collection and subsequent measurements were conducted blind to treatment. We measured two key indicators of ejaculate quality: total sperm count and sperm velocity (sperm swimming speed).

For total sperm counts, we vortexed the sample for ~1 min and then pipetted the solution 10-20 times to disperse sperm cells. We then pipetted 3 μL onto a 20 μm capillary slide (Leja), and used a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) to count sperm under x100 magnification. Threshold values defining cell detection were predetermined as elongation percentage 15-65 and head size 5-15 μm (Vega-Trejo et al. 2019; Harrison et al. 2023). We randomly counted five subsamples per sperm sample. These subsample counts were highly repeatable (R = 0.90; 95% CIs: 0.86, 0.93; P <0.001; from the R package *rpt*R (Stoffel et al. 2017)), so we used the average value per male for further analyses. We then estimated total sperm counts by adding the average sperm number per bundle (Vega-Trejo et al. 2016) to account for the six bundles removed for sperm velocity analyses.

To measure sperm velocity, we took two samples of three sperm bundles from each male's ejaculate and pipetted the bundles from each sample into two separate PCR tubes containing 2 μL extender medium. We pipetted each sample onto 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). Sperm was then 'activated' with 3 μL of activator solution (125 mM KCL and 2 mg/mL bovine serum albumin) and covered with a coverslip. We used a CEROS Sperm Tracker to record two measures of sperm velocity: VAP (average path velocity) and VCL (curvilinear velocity). As VAP and VCL were highly correlated (r = 0.97; 95% CIs = 0.96, 0.98; P <0.0001; n = 97 males), we used VCL for further analyses as it is more biologically relevant. All sperm velocity measures were taken immediately following sperm activation.

Relative telomere length

Focal males were euthanized following their final sperm stripping and their tails removed and stored in 80% ethanol at -20°C until ready for DNA extraction. We used a commercial tissue DNA

extraction kit (Monarch® Genomic DNA Purification Kit, New England BioLabs, Australia) to extract and purify genomic DNA from the tail muscle tissue. Prior to lysis, tail tissue (excluding the fin) was cut into smaller pieces and each sample was left in the lysis buffer mixture overnight (~18 hours) on an Eppendorf ThermoMixer set at 56°C and maximum mixing speed. Genomic DNA was concentrated with 70 μL elution buffer and quantitated with a Qubit fluorometer prior to dilution to 20 ng/μL with 10 mM Tris. In total, we extracted sufficient genomic DNA from *n* = 137 males.

Relative telomere length (rTL) was measured using real-time quantitative PCR (Cawthon 2002), determined as the ratio (T/S) of telomere repeat length (T) to a single-copy reference gene length (S). This ratio is proportional to average telomere length (Cawthon 2002). We used the standard telomere primers Tel1b (5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3') as described by (Criscuolo et al. 2009). Following previous studies that have measured teleost fish telomeres (Gao and Munch 2015; Monteforte et al. 2020), we used a *Gambusia*-specific region of the melanocortin 1 receptor (MC1R) as our control single-copy reference gene (see Supplementary Material for full details of qPCR tests and protocol). Our chosen MC1R primers were MC1R.F (5'-CCTGTAGGCGTAGATGAGCG-3') and MC1R.R (5'-CACCAGTCCCTTCTGCAACT-3').

We ran qPCRs in triplicate using 96-well plates. Telomere and MC1R amplifications were run concurrently on separate plates using QuantStudio3 (Thermo Fisher Scientific, Waltham, USA). For a given male, we first ran telomere qPCRs before immediately running MC1R qPCRs with each sample in the same corresponding well position across plates to minimise variation. We used 5 μL PowerUp™ SYBR™ Green Master Mix with 300 nM of both forward and reverse primers (9 μL total volume) and 1 μL of 20 ng/μL DNA extract to bring the total volume in each well to 10

293 μL. The qPCR cycling profile for MC1R started at 95°C for 3 min for denaturation, followed by
294 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 20 s for amplification. For telomeres,
295 denaturation started at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, then
296 72°C for 15 s. Both profiles had a final cycle (15 s at 95°C, 1 min at 60°C, and 15 sec at 95°C) that
297 generated melt curves to confirm qPCR specificity. Each plate had three negative controls (9 μL
298 reagent mix and 1 μL MilliQ purified water), two inter-plate control samples (run in triplicate), and
299 a golden sample (also in triplicate) at five DNA concentrations (0.05, 0.2, 1, 5 and 20 ng/μL) to
300 generate the standard curve and determine the amplification efficiency of each qPCR plate
301 (telomere: 1.99-2.11; MC1R: 1.93-2.01). The inter-plate controls were two individuals that had a
302 high amount of genomic DNA such that they could be run in triplicate across multiple plates. A
303 golden sample (i.e., the sample with the highest amount of genomic DNA and different from the
304 two inter-plate control individuals) was used in triplicate on each plate at five DNA concentrations
305 (0.05, 0.2, 1, 5 and 20 ng/μL) to generate the standard curve and determine the amplification
306 efficiency of each qPCR plate. The replicate samples from males were highly repeatable within each
307 qPCR plate (telomere: R = 0.77, SE = 0.03, $P < 0.0001$; MC1R: R = 0.95, SE = 0.01, $P < 0.0001$).
308
309 Relative telomere length was obtained using the equation (Pfaffl 2001):

310 $$rTL = \frac{E_{telomere}^{CqTelomere(interplate\ control)-CqTelomere(sample)}}{E_{MC1R}^{CqMC1R(interplate\ control)-CqMC1R(sample)}}$$

311 *(Eq. 1)*
312
313 Where $E_{telomere}$ is the mean efficiency of the telomere plate; E_{MC1R} is the mean efficiency of the MC1R
314 plate; *CqTelomere(interplate control)* and *CqMC1R(interplate control)* are the mean Cq (cycle
315 quantification) values of the average of the two reference DNA samples for telomere and MC1R

316 plates, respectively; and *CqTelomere(sample)* and *CqMC1R(sample)* are the mean Cq values from the
317 triplicate of each sample in the plate for telomere and MC1R, respectively.
318
319 *Statistical analyses*
320 We fitted generalised linear mixed models (GLMMs) to test if winners and losers differ in: a) their
321 investment into mating effort and ejaculate traits, b) telomere length, and c) whether reproductive
322 investment had an additive or interactive effect (i.e. differs for winners and losers) on each set of
323 traits.
324
325 For the three pre-copulatory behavioural traits (number of mating attempts, number of successful
326 attempts, time spent with the female), we ran separate GLMMs with negative binomial error
327 distributions (log-link functions). Our full models had contest outcome (winner/loser),
328 reproductive treatment (contests only/contests and mating), male size, and all three-way and two-
329 way interactions as fixed factors. Female size was included as a covariate (no interaction terms) and
330 block number (Block ID) as a random effect. Similarly, for ejaculate traits, we first fit GLMMs with
331 Gaussian error distributions (identity-link functions) with contest outcome, reproductive
332 treatment, male size, and their three-way and two-way interactions as fixed factors, with block
333 number as a random effect. For growth, we fit a GLMM with a Gaussian error distribution with
334 male size at the end of the treatment as the response variable. Contest experience and reproduction
335 treatment, and their interaction, were set as fixed factors. Initial male size (standardised and
336 centred) was included as a covariate (no interaction term), with block number as a random effect.
337 For relative telomere length, we fit a GLMM with a Gaussian error distribution with contest
338 outcome, reproductive treatment, male size, and their three-way interactions as fixed factors, with
339 block number as a random effect. However, model diagnostics and visual assessment of the model
340 residuals revealed three outliers (rTL > 1.9 and all three data points had standardised residuals with

a value >2.5) that were potentially influencing the model. We present the models with and without these outliers for transparency. However, we cannot determine the cause of these outliers, so we only interpret the model in which they are included. There was no obvious sign that these outliers were due to measurement error (e.g., they were from different qPCR plates and did not have unusually high Cq values for either their telomeres or their MC1R gene). Regardless of whether these outliers were excluded or included, male size had a significant effect on telomere length, but their exclusion resulted in the effect of winning/losing contest treatment becoming statistically significant.

Finally, we ran *post hoc* tests for significant correlations among the seven reproduction and life-history traits that we measured. The correlations for each of the four types of males are not referred to in the main text but are in the Supplementary Material (Tables S2-S5) for interested readers.

All statistical analyses were conducted using R version 4.0.2 (R Development Core Team 2020). We used the package *glmmTMB* (Brooks et al. 2017) to initially fit GLMMs with several different error distributions (Gaussian, Poisson, negative binomial and zero-inflated Poisson) and link functions (log for Poisson, negative binomial and zero-inflated Poisson distributions, identity for Gaussian distributions) then used Akaike Information Criteria (AIC) tables to identify the best-fitting model. We used the *DHARMa* package (Hartig 2020) to run model diagnostics. Where interaction terms were not significant, they were removed from the model to quantify main effects. For reduced models, we obtained significance of fixed effects from ANOVA type II Wald chi-square (χ^2) tests and type III for models with interactions. Pairwise comparisons and their significance as shown in Figures are from *t*-tests using the package *ggpubr* (Kassambara 2020). These pairwise comparisons were conducted for ease of visualisation and not data interpretation, so we did not correct for multiple testing. We set $\alpha = 0.05$ for all model terms except for three-way

interaction terms (where α was 0.01). All tests were two-tailed. Descriptive statistics for each trait are shown in Table 1.

Results

Pre-copulatory reproductive investment

On average, winners made significantly more mating attempts than losers when males had not previously been able to mate, but there was no winner-loser difference when males had previously had full access to females; and winners made significantly fewer mating attempts when they had previously been able to mate than when they had not (Figure 2A). Winners spent significantly more time than losers associating with the female ($\chi^2 = 28.45$, df = 1, $P < 0.0001$; Table 2), but they were not more successful at mating ($\chi^2 = 1.97$, df = 1, $P = 0.160$; Table 2; Figure 2B & C). Males that had previously been able to mate made significantly fewer successful mating attempts ($\chi^2 = 5.99$, df = 1, $P = 0.014$), and spent far less time associating with females ($\chi^2 = 27.10$, df = 1, $P < 0.0001$), than males that had not had full access to females (Table 2).

Only one of the three pre-copulatory traits we measured showed an interaction between past contest experience and reproductive opportunity: there was a significant three-way interaction between male size, contest experience and reproductive opportunity affecting the number of mating attempts ($\chi^2 = 10.44$, df = 1, $P = 0.001$; Table 2). The number of mating attempts increased with male body size for winners when males had previously been able to mate but decreased if they had not; and the reverse pattern occurred for losers (Figure 3A). Neither male nor female body size significantly affected the number of successful attempts made by males (male size: $\chi^2 = 2.37$, df = 1, $P = 0.124$; female size: $\chi^2 = 1.69$, df = 1, $P = 0.194$), nor the time spent near the female (male size: $\chi^2 = 0.45$, df = 1, $P = 0.501$; female size: $\chi^2 = 0.92$, df = 1, $P = 0.338$).

392 *Post-copulatory reproductive investment*

393 Contrary to our expectations, there were no significant interactions between past contest
394 experience and the past opportunity to mate that affected either sperm counts or sperm velocity
395 (Table 2). Winners and losers did not significantly differ in either their sperm count ($\chi^2 = 0.06$, df
396 = 1, $P = 0.804$; Figure 3B) or sperm velocity ($\chi^2 = 0.94$, df = 1, $P = 0.333$; Figure 3C). As expected,
397 however, larger males had a higher total sperm count ($\chi^2 = 4.00$, df = 1, $P = 0.047$; Figure 3B).
398
399 Males that had previously had or had not been able to mate did not differ in their sperm count (χ^2
400 = 0.30, df = 1, $P = 0.582$; Figure 3B), but there was a significant interaction with male size that
401 affected sperm velocity ($\chi^2 = 7.97$, df = 1, $P = 0.005$). Larger males had faster swimming sperm
402 when they had previously been able to mate, but there was no effect of body size when males had
403 not previously had full access to females (Figure 3C).
404
405 *Growth*
406 After nine weeks, males that had previously been able to mate grew significantly less than males
407 that had not had full access to females ($\chi^2 = 5.62$, df = 1, $P = 0.018$). In contrast, winning or losing
408 did not affect male growth ($\chi^2 = 1.51$, df = 1, $P = 0.219$) (Table 2; Figure 3D).
409
410 *Relative telomere length*
411 There were no significant interactions between past contest experience and the past opportunity
412 to mate affecting relative telomere length (Table 2); and neither past contest experience ($\chi^2 = 1.52$,
413 df = 1, $P = 0.218$; but see Methods and Table 2 for the findings after removing three statistical
414 outliers) nor the past opportunity to mate ($\chi^2 = 0.00$, df = 1, $P = 0.958$) affected relative telomere
415 length. However, larger males had significantly longer telomeres than smaller males, regardless of
416 their treatment ($\chi^2 = 5.50$, df = 1, $P = 0.019$; Figure 4A, B).

Mortality

Of the initial 176 focal males, 34 did not survive to the end of the 9-week treatment period (~83% survival). We ran a *post hoc* test to test if treatment type affected mortality (i.e., this test was not explicitly mentioned in our OSF pre-registration). We ran a Cox proportional hazards regression with contest experience, reproductive opportunity, their two-way interaction, and male body size treated as fixed factors. We then removed the non-significant interaction between contest experience and reproduction treatment and reran the model with only the main effects. Being a winner (coefficient = -0.92, SE = 0.40, z = -2.44, P = 0.015), or a larger male (coefficient = -0.39, SE = 0.68, z = -0.39, P = 0.041), significantly increased survival. Interestingly, however, mortality did not significantly differ between males that did or did not have the opportunity to mate (coefficient = 0.06, SE = 0.34, z = 0.06, P = 0.857).

Discussion

We used a 2 x 2 experimental design to test how long-term winning or losing, in combination with variation in past reproductive effort, influenced telomere length and several key life-history traits in the Eastern mosquitofish, *Gambusia holbrooki*. After 9 weeks there were significant differences between winners and losers in their pre-copulatory reproductive investment (i.e., mating effort), but not in their post-copulatory reproductive investment (i.e., ejaculates). There was no evidence that consistently winning or losing affected body growth, and there was only weak evidence (i.e., statistically significant only if three outliers were removed) that winners had shorter telomeres than losers. Interestingly, males that had previously had full access to females (i.e., greater past reproductive investment) had significantly lower current mating effort, lower body growth, and slower swimming sperm, but only if they were small bodied. Again, however, there was no detectable effect on telomere length. Taken together, our results show that: 1) losing contests and

greater past reproductive effort seems to lower current mating effort, 2) winning contests and greater past reproductive effort does not detectably accelerate telomere loss (hence lower expected lifespan), and 3) significant interactions with male size suggest that there are size-dependent costs of past reproductive effort and winning/losing fights that affect current reproductive effort, but not growth or telomere shortening.

Prior contest experience and current mating effort

Winners had significantly better pre-copulatory performance than losers but there were no significant differences in their post-copulatory reproductive investment. That is, winners made more mating attempts and spent more time with the female than did losers but did not differ in their sperm counts or sperm velocity. These results broadly replicate those of an earlier study that tested for a long-term winner-loser effect on the plasticity of male investment into pre- and post-copulatory sexually selected traits (Harrison et al. 2023). Surprisingly, however, in the current study we found no differences between winners and losers in how many successful mating attempts they made. In the earlier study, males experienced only winning or only losing against a rival male in the presence of a female (i.e., males could perceive but not mate with a female). This experimental design is comparable to our 'contests only' treatment in the current study. It is therefore worth noting that when we directly compare winners and losers from the 'contests only' treatment, winners did make significantly more successful mating attempts than losers (see Figure 2B). Additionally, the earlier study quantified pre-copulatory mating effort when size-matched winners and losers directly competed for a female. In the present study, we instead quantified male mating effort in the absence of a rival. It seems plausible that direct interactions between winning and losing males influence their pre-copulatory success, especially where winners monopolise access to a female. The difference between the two studies therefore helps clarify the mechanism driving the previous findings (Harrison et al. 2018, 2023).

Past reproductive investment: the costs of pre- and post-copulatory investment

Greater male reproductive effort is generally associated with decreased somatic maintenance, hence reduced lifespan. There is evidence from several species that the energetic costs of mating effort (i.e., courtship) are sufficient to reduce somatic maintenance and lifespan (Cordts and Partridge 1996; Martin and Hosken 2004). But very few studies have quantified the relative costs of male mating effort and ejaculate production as the two processes usually co-occur. This has, however, recently been done for *Gambusia holbrooki*: the costs of pre-copulatory mating effort are significantly greater than those due to ejaculation (Chung et al. 2021). In our study, we corroborated the additional fitness costs of sperm production by comparing males that did or did not have the opportunity to mate with females and, hence, the opportunity to replenish sperm. Males that had previously been able to mate had lower pre-copulatory success (less time harassing females, and fewer successful copulation attempts) than males that were unable to mate. Males that were previously able to mate also had significantly lower growth rates. There was therefore a detectable cost of sperm production on both sexually and naturally selected traits. Intriguingly, however, there was no detectable effect on sperm quantity; and the effect on sperm velocity depended on male size, but with the unexpected result that males who had previously been able to mate, hence replenish sperm, had *faster* swimming sperm, but only if they were larger males (see Figure 3C). While Chung *et al.* (2021) demonstrated that pre-copulatory mating effort is relatively more expensive than post-copulatory traits, a key point of difference in our study is that males consistently encountered novel females *and* unfamiliar rivals. Focal males were always fighting with a new rival, either in the presence of a female (contests only) or directly for mating opportunities (contests and reproduction) for 9 weeks. We found that when males experienced consistent sperm competition, and interacted with new rivals for unfamiliar females, the effort of maintaining competitive ejaculates came at the cost of reduced current mating effort and slower growth. This

might partially be due to trade-offs between investment into ejaculate competitiveness and somatic maintenance (Lemaître et al., 2020b).

It is intriguing that males that could mate, hence allocate more to sperm production during the experimental treatment, thereafter had reduced pre-copulatory effort but no detectable decline in ejaculate quality. Males are expected to increase their relative investment into pre-copulatory mating effort when such traits reduce sperm competition risk (i.e., mate guarding or harassment; (Lüpold et al. 2014)). Trade-offs between different components of male reproductive investment can therefore occur when males plastically adjust their mating effort to counter changes in perceived sperm competition risk, or when they more frequently encounter unfamiliar females (Dewsbury 1981). For example, in an experimental manipulation of male reproductive effort, male guppies (*Poecilia reticulata*) that encountered novel females every four days over four months increased their sperm production but made fewer courtship displays than males that only encountered unfamiliar females every ten days (Devigili et al. 2015). Males that have previously invested heavily into reproduction might maximise their future reproductive success by reallocating their investment into cheaper reproductive traits, which could potentially explain age-dependent changes in relative investment into mating effort and sperm production (Gasparini et al., 2019). In our study, males that could fully invest into reproduction had slower growth than males that did not, suggesting that relative investment into reproduction reduces investment into somatic maintenance, but not lifespan (i.e., telomere shortening). The combination of fewer resources to invest and a shift in relative investment could produce the pattern we observed in *G. holbrooki*; lower mating effort but no change in ejaculates when comparing males with a difference in past reproductive effort.

Telomeres

Unexpectedly, we did not find a significant difference in the telomere lengths of males with full or no access to females. However, while reproduction is assumed to be energetically costly, there is equivocal evidence that it increases oxidative damage via oxidative stress (reviewed in Speakman & Garratt, 2013). Similarly, while the outcomes of male-male contests can induce different stress responses in winners and losers, we found equivocal evidence that winners have greater telomere loss than losers. One potential explanation is that the immune and/or endocrine system compensates when exposure to the same stressful event is sustained for a long period. Sudden changes in social status can trigger oxidative stress (Beaulieu et al. 2014), but once dominance hierarchies stabilise and agonistic interactions decrease, then the immune and endocrine systems adjust to match the new group dynamics (Milewski et al. 2022). For example, newly dominant male East African cichlids (*Astatotilapia burtoni*) initially have a lower ability to mitigate oxidative damage, but after 14 days show similar oxidative stress markers to those in established males that had retained their dominance status over this period (Fialkowski et al. 2021). In our experimental study, focal male *G. holbrooki* were unlikely to have established stable dominance hierarchies as they faced a new rival every ~2 days. It is therefore more plausible that the experience of always winning or losing fights imposes different types of costs, but ones that induce similar levels of oxidative stress and telomere shortening (Costanzo et al. 2021). For example, high levels of testosterone (associated with winning) or stress hormones (associated with losing) can both induce oxidative damage. Intriguingly, in our study, winners had lower mortality rates than losers, but with a trend towards shorter telomeres. This mortality difference raises the possibility of a selection bias towards measuring losers that could better withstand a stressful competitive environment, who potentially had longer telomeres than those that died during the 9-week experimental period. Finally, it is noteworthy that losers had *longer* telomeres than winners if we removed three outliers. Given that our results test for a relationship between past winning/losing experiences and telomere length, *independent* of initial male condition, this topic clearly warrants further investigation.

Body size

Finally, how individuals respond to stress and manage oxidative damage seems to be partially mediated by body size. Smaller males had both higher mortality and significantly shorter telomeres than large males. The latter finding is particularly unexpected given that large body size is often negatively correlated with telomere length (e.g., Ringsby et al., 2015). In mammals, a negative relationship between body size and telomere length is thought to be an adaptive response to the increased cancer risk associated with increased cellular replication (Pepke and Eisenberg 2021) such that larger individuals suppress telomerase activity or have relatively longer telomeres at birth (reviewed in: Risques & Promislow, 2018). However, the relationship between telomere length and body size is not negative in all taxa (Monaghan and Ozanne 2018), and telomerase activity is much more variable in ectotherms than endotherms (Olsson et al. 2018). It is possible that small-bodied *G. holbrooki* in our study lacked the energetic resources necessary to engage in prolonged male-male competition, regardless of whether they consistently won or lost, thereby elevating mortality. Taken together, our findings suggest that large males are better able to manage the costs associated with competition and reproduction, although the mechanism remains unclear.

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Author Contributions

LMH conceived and designed the experiment under the supervision of MDJ. LMH conducted experiments and collected data. OS and LMH conducted molecular lab work. LMH conducted statistical analyses and wrote the first draft of the manuscript. OS and MDJ provided critical revisions. This research was supported by an Australian Research Council (ARC) Discovery Grant awarded to MDJ (DP190100279).

Data Accessibility Statement

All data and code are provided as Supplementary Material for review. Data and code will be deposited at the Dryad Repository upon acceptance and will be made freely available through our pre-registration on the OSF: https://osf.io/saj46/

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Figures and Tables

Table 1. Descriptive statistics for each of the behavioural, ejaculate, and life-history traits.

	Winners						**Losers**					
	Contests Only			Contests + Reproduction			Contests Only			Contests + Reproduction		
Trait	*Mean*	*SD*	*n*	*Mean*	*SD*	*n*	*Mean*	*SD*	*n*	*Mean*	*SD*	*n*
Mating attempts	26.27	19.17	41	18.11	17.20	37	19.55	20.88	31	14.55	12.60	33
Successes	2.49	2.40	41	1.11	1.58	37	1.35	1.85	31	1.24	1.71	33
Time with female (s)	438.31	218.15	41	241.54	148.23	37	248.12	242.52	31	125.73	78.00	33
Sperm count	2086739	1926763	32	1820071	1675049	29	2428632	2700767	25	1974701	1502069	25
Sperm velocity ($\mu m/s^{-1}$)	165.13	22.10	29	166.28	23.54	25	155.36	22.23	21	167.60	24.69	21
Initial male size (mm)	20.34	1.59	41	20.57	1.55	37	20.58	1.63	31	20.27	1.42	33
Growth*† (mm)	-0.62	0.51	39	-0.46	0.48	37	-0.56	0.41	31	-0.33	0.33	33
Relative telomere length	0.87	0.45	39	0.82	0.43	36	0.90	0.45	31	0.95	0.36	31

* two outliers removed from winners (contests only) treatment group. Outliers can be observed in Figure 4A.

† growth calculated as final male size – initial male size

Table 2. Model estimates from the generalised linear mixed models for each of the reproduction and life-history traits measured. Significant effects are highlighted in bold. The estimate is for the level of the factor shown in parentheses.

Model Parameters	Estimate	SE	z	*P*-value
1) Number of mating attempts‡				
Intercept	**2.58**	**0.16**	**15.86**	**<0.0001**
Male size (Centred and standardised)	-0.32	0.17	-1.87	0.062
Experience (Winning)	0.23	0.22	1.02	0.308
Treatment (Contests only)	0.34	0.23	1.47	0.141
Female size (Centred and standardised)	0.11	0.09	1.28	0.200
Male size x Experience (Winning)	**0.59**	**0.22**	**2.60**	**0.009**
Male size x Treatment (Contests only)	**0.60**	**0.23**	**2.63**	**0.009**
Experience (Winning) x Treatment (Contests only)	0.12	0.31	0.40	0.691
Male size x Experience x Treatment	**-0.97**	**0.30**	**-3.23**	**0.001**
2) Number of successful mating attempts§				
Intercept	-0.04	0.20	-0.19	0.849
Male size (Centred and standardised)	-0.18	0.12	-1.54	0.124
Experience (Winning)	0.29	0.21	1.40	0.161
Treatment (Contests only)	**0.50**	**0.21**	**2.45**	**0.014**
Female size (Centred and standardised)	0.14	0.11	1.30	0.194
3) Time with female (seconds) §				
Intercept	**4.80**	**0.13**	**36.56**	**<0.0001**
Male size (Centred and standardised)	0.05	0.08	0.67	0.501
Experience (Winning)	**0.66**	**0.12**	**5.33**	**<0.0001**
Treatment (Contests only)	**0.64**	**0.12**	**5.21**	**<0.0001**
Female size (Centred and standardised)	0.07	0.07	0.96	0.337
4) Total sperm count (log transformed) §				
Intercept	**13.95**	**0.25**	**56.92**	**<0.0001**
Male size (Centred and standardised)	**0.31**	**0.16**	**1.99**	**0.047**
Experience (Winning)	-0.05	0.22	-0.25	0.804
Treatment (Contests only)	0.12	0.22	0.55	0.582
5) Sperm velocity (VCL) §				
Intercept	**164.30**	**4.76**	**34.51**	**<0.0001**
Male size (Centred and standardised)	6.16	4.49	1.37	0.170
Experience (Winning)	4.05	4.18	0.97	0.333
Treatment (Contests only)	-5.63	4.21	-1.34	0.181
Male size x Treatment (Contests only)	**-12.49**	**4.43**	**-2.82**	**0.005**
6) Growth (final size, mm) §				
Intercept	**2.91**	**0.59**	**4.96**	**<0.0001**
Initial male size (Centred and standardised)	**0.87**	**0.03**	**30.64**	**<0.0001**
Experience (Winning)	0.11	0.09	1.23	0.219
Treatment (Contests only)	**0.21**	**0.09**	**2.37**	**0.018**
7) Relative telomere length (rTL) – with outliers§				
Intercept	**0.92**	**0.07**	**14.17**	**<0.0001**
Male size (Centred and standardised)	**0.09**	**0.04**	**2.35**	**0.019**
Experience (Winning)	-0.09	0.07	-1.23	0.218
Treatment (Contests only)	0.00	0.07	0.05	0.958

8) Relative telomere length (rTL) – without 3 outliers§				
Intercept	**0.93**	**0.06**	**16.44**	**<0.0001**
Male size (Centred and standardised)	**0.10**	**0.03**	**3.15**	**0.002**
Experience (Winning)	**-0.13**	**0.06**	**-2.07**	**0.038**
Treatment (Contests only)	-0.01	0.06	-0.18	0.859

‡ full model is shown for mating attempts where there is a significant three-way interaction.

§ reduced models presented. See Supplementary Material for full models.

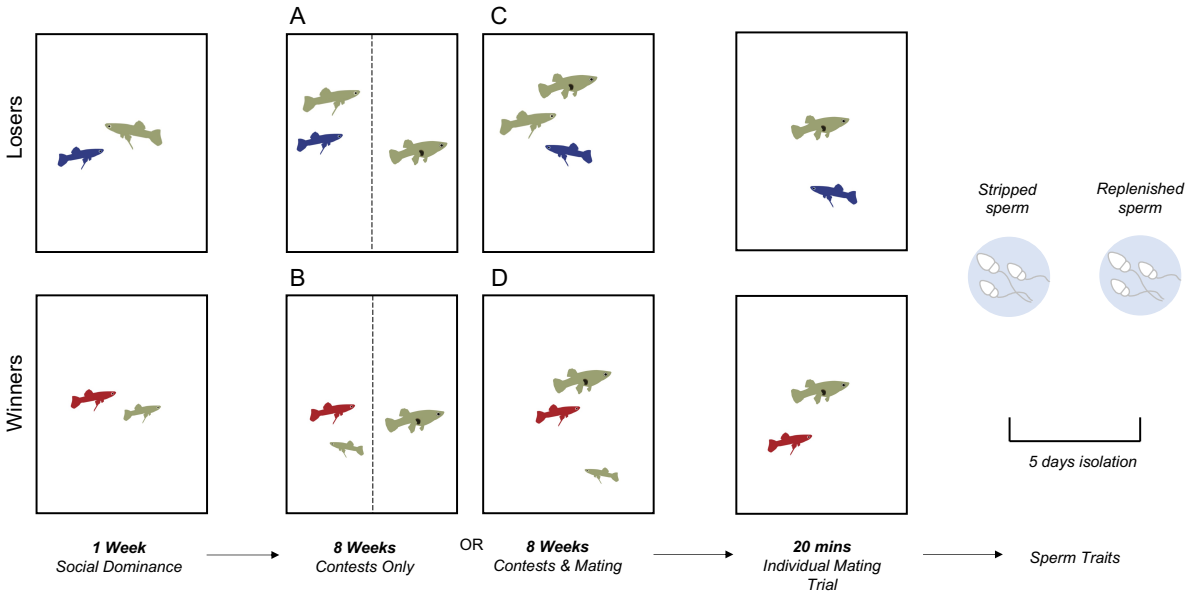

Figure 1. Experimental design to create winners and losers. Following 1 week of isolation, focal males (winners in red; losers in blue) were randomly assigned to become either a loser or winner by being paired with a larger or smaller rival, respectively. Males had 1 week of contests facing new rivals daily to establish social dominance. Focal males were then randomly assigned to experience either only male-male contests (A and B) or male-male contests with the opportunity to mate (C and D) for a further 8 weeks. Rival males and females were rotated every few days. At the end of 9 weeks, males were placed with a random adult female to quantify their mating behaviour during individual mating trials. They were then immediately stripped of their sperm. After 5 days in isolation, focal males were again stripped of their sperm to measure their replenished sperm traits before being euthanized to measure relative telomere length.

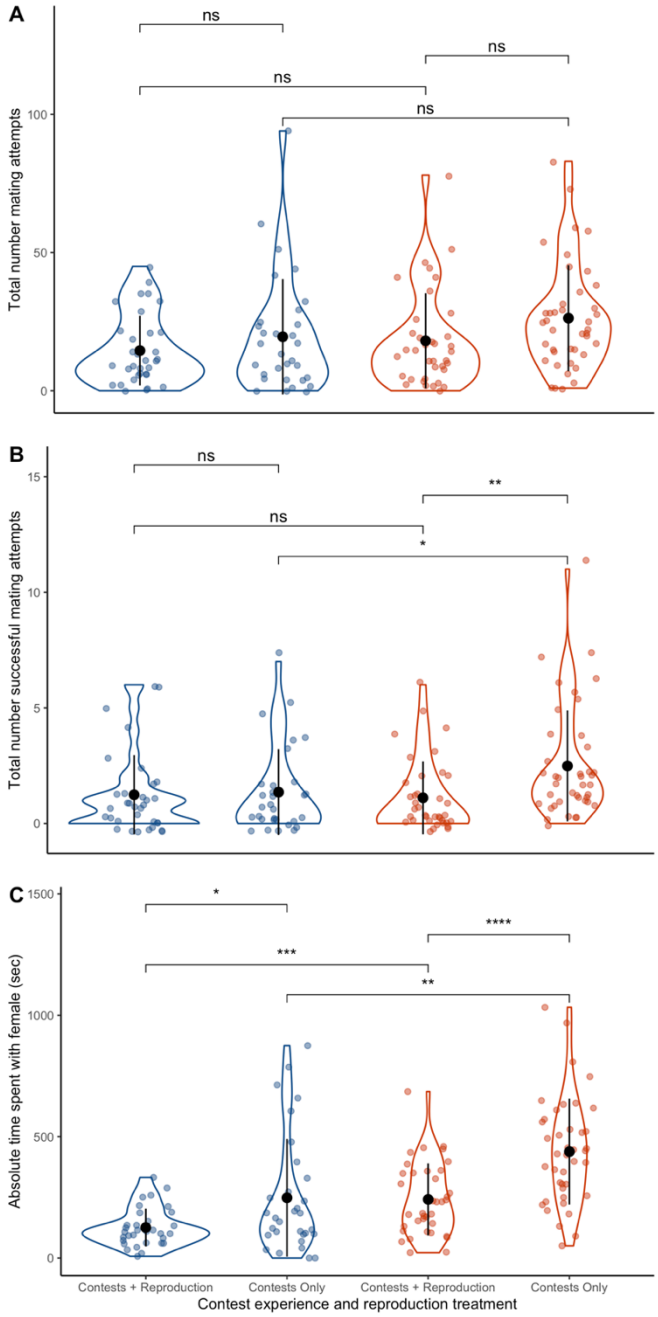

Figure 2. Violin plots depicting the pre-copulatory investment of winners (red) and losers (blue) when males had either no access (Contests Only) or full access to mating opportunities (Contests + Reproduction) for nine weeks. Asterisks (*) indicate significant pairwise differences between winners and losers within each reproduction treatment or between reproduction treatments for either winners or losers (ns = no significant difference). Mean and standard deviation shown in black.

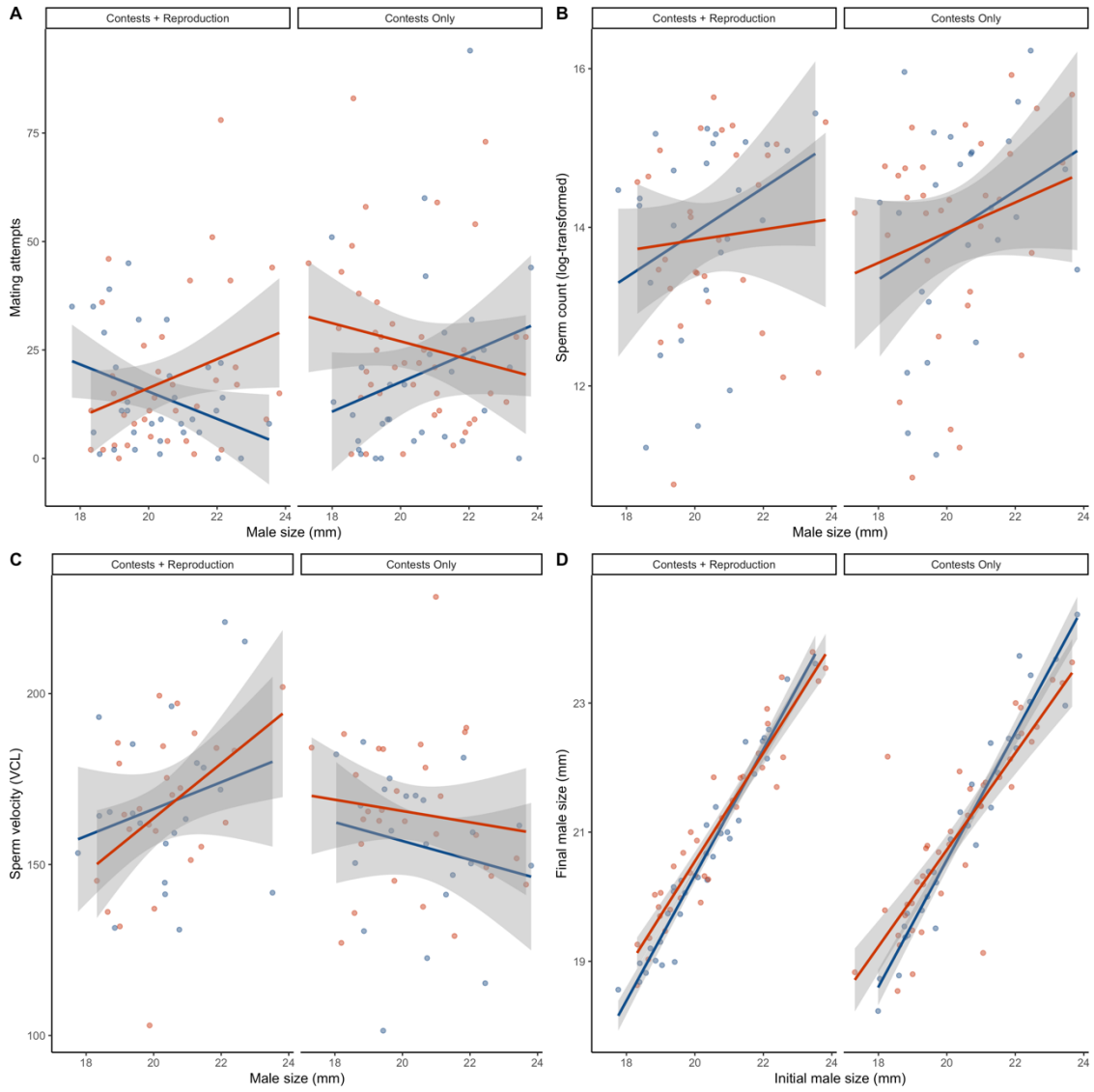

Figure 3. Simple linear regressions with 95% confidence intervals (grey ribbons) highlight interactions between male body size and several key reproductive traits: the number of mating attempts (A), total sperm counts (B), and sperm velocity (C). After nine weeks, winners (red) and losers (blue) differed in the number of mating attempts made (A); large winners that had access to mates (Contests + Reproduction) made more mating attempts than large winners that had no access (Contests Only). Larger males produced more sperm than did smaller males regardless of treatment (B), while larger males produced faster sperm than smaller males, but only for males that had full access to mates (C). Males without full access to females (Contests Only) grew significantly faster than males that could mate (Contests + Reproduction) (D).

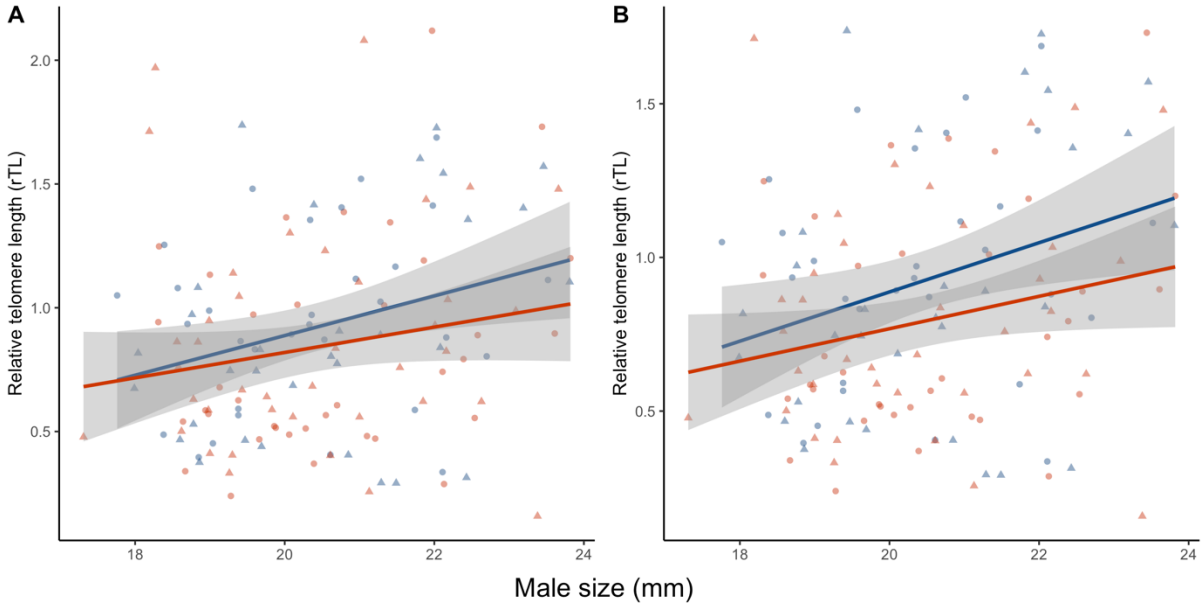

Figure 4. Simple linear regressions with 95% confidence intervals (grey ribbons) highlight the relationships between male body size and relative telomere length when outliers were present (A) or removed (B). Larger males had significantly longer telomeres than smaller males (A & B), while there was a trend for winners (red) to have shorter telomeres than losers (blue) when outliers were removed (B). Contests Only males are represented by triangles; Contests + Reproduction males are represented by circles.

Supplementary Material

1. qPCR test of MC1R primers

We initially planned to use GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as our single-copy reference gene (as used previously for *G. holbrooki* by Rollings *et al.*, 2014). However, we could not obtain sufficient GAPDH amplicons during our initial qPCR trials and we subsequently observed duplication of the GAPDH gene in the genome of the close relative, *Gambusia affinis*. Further mapping of the *G. affinis* genome identified three potential primer pairs that would recognise a single region corresponding to the MC1R gene.

We constructed a BLAST database from the *Gambusia affinis* reference genome assembly SWU_Gaff_1.0 (GCA_019740435.1) (Shao et al. 2020) and searched the database for all publicly available coding sequence accessions in GenBank for the melanocortin 1 receptor (MC1R) gene in bony fishes (Actinopterygii). Hits fell into one of two regions, one of which had lower identities and shorter matches to the input sequences than the other. We discarded hits to the former region and merged the mapped locations of all remaining hits into a 968 bp interval on linkage group 2 (NC_057869.1). We extracted this region from the assembly as a fasta file and used it as input to GenBank's Primer-BLAST tool, specifying a PCR amplicon of 100-200 bp and leaving all other parameters as default. We did not specify specific regions for the placement of forward and reverse primers. We selected the top 3 pairs of primers and then used BLAST again to check their specificity against the reference genome. Curiously, BLAST found no matches in the reference genome sequence when run locally using our own BLAST database, despite these primers being designed from that very sequence. We instead used GenBank's online BLAST platform to search the reference genome, and found the best, full-length hit to the expected location on linkage group 2. For each primer, there were other hits detected, but these were all of substantially higher E-value, lower identity, and shorter alignment length than the best hit.

We tested the three primer pairs at three different annealing temperatures (57°C, 60°C and 63°C). We tested two different gDNA samples and a blank (water), and ran each combination of sample, primer pair, and annealing temperature in duplicate. We used 5 μL PowerUp™ SYBR™ Green Master Mix with 300 nM of both forward and reverse primers (9 μL total volume) and 1

μL of 20 ng/μL DNA extract (or water for blanks) to bring the total volume in each well to 10 μL.

We tested the three MC1R primer pairs using a qPCR cycling profile that started at 95°C for 3 min for denaturation, followed by 40 cycles of 95°C for 15 s, (57°C, 60°C or 63°C) for 30 s, and 72°C for 20 s for amplification, and a final cycle (15 s at 95°C, 1 min at 60°C, and 15 sec at 95°C) that generated melt curves to confirm qPCR specificity. After tests, our chosen MC1R primers were MC1R.F (5'-CCTGTAGGCGTAGATGAGCG-3') and MC1R.R (5'-CACCAGTCCCTTCTGCAACT-3') at an annealing temperature of 60°C.

2. *Final plate layout for telomeres and MC1R qPCR*

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	S2	S2	S2	S3	S3	S3	Standard curve 20ng	Standard curve 20ng	Standard curve 20ng
B	S4	S4	S4	S5	S5	S5	S6	S6	S6	Standard curve 5ng	Standard curve 5ng	Standard curve 5ng
C	S7	S7	S7	S8	S8	S8	S9	S9	S9	Standard curve 1ng	Standard curve 1ng	Standard curve 1ng
D	S10	S10	S10	S11	S11	S11	S12	S12	S12	Standard curve 0.2ng	Standard curve 0.2ng	Standard curve 0.2ng
E	S13	S13	S13	S14	S14	S14	S15	S15	S15	Standard curve 0.05ng	Standard curve 0.05ng	Standard curve 0.05ng
F	S16	S16	S16	S17	S17	S17	S18	S18	S18	Golden Sample S1	Golden Sample S2	-ve
G	S19	S19	S19	S20	S20	S20	S21	S21	S21	Golden Sample S1	Golden Sample S2	-ve
H	S22	S22	S22	S23	S23	S23	S24	S24	S24	Golden Sample S1	Golden Sample S2	-ve

885 *3. Full model summaries for each of the seven traits*
886
887 **Table S1.** Model estimates from the full generalised linear mixed models for each of the
888 reproduction and life-history traits measured. Significant effects are highlighted in bold (alpha =
889 0.01 for 3-way interactions). The estimate is for the level of the factor shown in parentheses.

Model Parameters	Estimate	SE	χ	*P*-value
9) Number of mating attempts				
Intercept	**2.58**	**0.16**	**15.86**	**<0.0001**
Male size (Centred and standardised)	-0.32	0.17	-1.87	0.062
Experience (Winning)	0.23	0.22	1.02	0.308
Treatment (Contests only)	0.34	0.23	1.47	0.141
Female size (Centred and standardised)	0.11	0.09	1.28	0.200
Male size x Experience (Winning)	**0.59**	**0.22**	**2.60**	**0.009**
Male size x Treatment (Contests only)	**0.60**	**0.23**	**2.63**	**0.009**
Experience (Winning) x Treatment (Contests only)	0.12	0.31	0.40	0.691
Male size x Experience x Treatment	**-0.97**	**0.30**	**-3.23**	**0.001**
10) Number of successful mating attempts				
Intercept	0.10	0.23	0.45	0.655
Male size (Centred and standardised)	-0.33	0.26	-1.27	0.206
Experience (Winning)	-0.04	0.30	-0.15	0.884
Treatment (Contests only)	0.11	0.31	0.36	0.718
Female size (Centred and standardised)	**0.17**	**0.10**	**1.65**	**0.099**
Male size x Experience (Winning)	0.31	0.33	0.91	0.361
Male size x Treatment (Contests only)	0.49	0.31	1.55	0.122
Experience (Winning) x Treatment (Contests only)	0.64	0.41	1.59	0.113
Male size x Experience x Treatment	-0.84	0.42	-2.01	0.045
11) Time spent with the female				
Intercept	**4.80**	**0.14**	**33.59**	**<0.0001**
Male size (Centred and standardised)	0.01	0.14	0.05	0.959
Experience (Winning)	**0.66**	**0.17**	**3.84**	**<0.001**
Treatment (Contests only)	**0.60**	**0.18**	**3.33**	**<0.001**
Female size (Centred and standardised)	0.07	0.07	0.98	0.328
Male size x Experience (Winning)	0.01	0.17	0.08	0.935
Male size x Treatment (Contests only)	0.24	0.17	1.39	0.165
Experience (Winning) x Treatment (Contests only)	0.03	0.24	0.11	0.912
Male size x Experience x Treatment	-0.35	0.23	-1.50	0.134
12) Sperm count (log-transformed)				
Intercept	**14.00**	**0.27**	**50.93**	**<0.0001**
Male size (Centred and standardised)	-0.11	0.31	-0.34	0.735
Experience (Winning)	0.04	0.32	0.12	0.904
Treatment (Contests only)	0.45	0.28	1.62	0.106
Male size x Experience (Winning)	0.11	0.43	0.25	0.804
Male size x Treatment (Contests only)	-0.36	0.34	-1.06	0.290
Experience (Winning) x Treatment (Contests only)	-0.02	0.34	-0.07	0.942
Male size x Experience x Treatment	0.27	0.45	0.60	0.545
13) Sperm velocity				
Intercept	**166.71**	**5.32**	**31.34**	**<0.0001**
Male size (Centred and standardised)	-0.36	6.02	-0.06	0.953
Experience (Winning)	-10.37	6.25	-1.66	0.097
Treatment (Contests only)	2.84	5.55	0.51	0.608
Male size x Experience (Winning)	8.63	8.34	1.03	0.301
Male size x Treatment (Contests only)	7.18	6.71	1.07	0.285
Experience (Winning) x Treatment (Contests only)	-9.86	6.28	-1.57	0.116
Male size x Experience x Treatment	-5.73	8.74	-0.66	0.512
14) Growth				

Intercept	**20.74**	**0.09**	**228.80**	**<0.0001**
Male size (Centred and standardised)	**1.34**	**0.04**	**30.52**	**<0.0001**
Experience (Winning)	0.17	0.12	1.34	0.181
Treatment (Contests only)	**0.27**	**0.13**	**2.08**	**0.037**
Male size x Experience (Winning)	-0.12	0.18	-0.66	0.506
15) Relative telomere length – with outliers				
Intercept	**0.96**	**0.07**	**12.82**	**<0.0001**
Male size (Centred and standardised)	0.08	0.08	0.98	0.327
Experience (Winning)	-0.16	0.10	-1.63	0.103
Treatment (Contests only)	-0.08	0.10	-0.77	0.444
Male size x Experience (Winning)	0.05	0.10	0.51	0.608
Male size x Treatment (Contests only)	0.08	0.10	0.77	0.443
Experience (Winning) x Treatment (Contests only)	0.15	0.14	1.06	0.288
Male size x Experience x Treatment	-0.19	0.14	-1.34	0.179
16) Relative telomere length – without outliers				
Intercept	**0.957**	**0.07**	**14.53**	**<0.0001**
Male size (Centred and standardised)	0.08	0.07	1.06	0.292
Experience (Winning)	**-0.19**	**0.09**	**-2.05**	**0.040**
Treatment (Contests only)	-0.07	0.09	-0.78	0.438
Male size x Experience (Winning)	0.03	0.09	0.35	0.730
Male size x Treatment (Contests only)	0.09	0.10	0.97	0.331
Experience (Winning) x Treatment (Contests only)	0.11	0.13	0.87	0.387
Male size x Experience x Treatment	-0.14	0.13	-1.10	0.274

4. Correlations between reproduction and life-history traits within males

We decided to run a *post hoc* test for significant correlations among the seven reproduction and life-history traits we measured. If males have similar levels of access to resources, a negative correlation hints at a trade-off. For each of the four types of males (2 reproduction treatments by 2 contest experience treatments) we generated a separate correlation matrix using only males with data for all seven traits. There were three significant negative correlations; prior losers that had free access to mating opportunities showed a significant negative correlation between relative telomere length and the number of successful mating attempts (r_s = -0.40, 95% CIs: -0.96, -0.02; P = 0.046, Table S3) and between relative telomere length and the time males spent with the female (r_s = -0.42, 95% CIs: -0.97, -0.21; P = 0.02). Similarly, prior losers that did not have free access to mating opportunities also showed a significant negative correlation between relative telomere length and the number of successful mating attempts males made (r_s = -0.38, 95% CIs: -0.98, -0.24; P = 0.018, Table S3). There were no other significant negative correlations between traits (Tables S2-S5). However, losers that had not previously had full access to females showed significant positive correlations between their number of mating attempts and the time spent near the female during mating trials (Losers, Contests Only: r_s = 0.81, 95% CIs: 0.52, 0.99; P =0.004; Table S4; Losers, Contests and Reproduction: r_s = 0.56, 95% CIs: 0.08, 0.97; P = 0.037; Table S5).

Table S2. Spearman's correlation coefficients with confidence intervals (in brackets) for each of the traits we measured for winning males that had no access to mating opportunities. Correlations were calculated for complete observations only (where males had all traits measured). Significant correlations indicated with asterisks (*).

Trait	*Mating attempts*	*Successful attempts*	*Time with female*	*Sperm count*	*Sperm velocity*	*Growth*	*rTL*
Mating attempts	-	0.29 (-0.43, 0.90)	0.46 (-0.02, 0.96)	-0.29 (-0.95, 0.12)	-0.18 (-0.89, 0.48)	-0.21 (-0.93, 0.27)	-0.04 (-0.83, 0.64)
Successful attempts	0.29 (-0.43, 0.90)	-	0.35 (-0.35, 0.92)	0.16 (-0.81, 0.69)	-0.26 (-0.92, 0.34)	-0.40 (-0.96, 0.07)	0.25 (-0.64, 0.83)
Time with female	0.46 (-0.02, 0.96)	0.35 (-0.35, 0.92)	-	-0.12 (-0.91, 0.40)	-0.08 (-0.86, 0.58)	-0.32 (-0.95, 0.10)	-0.18 (-0.89, 0.50)
Sperm count	-0.29 (-0.95, 0.12)	0.16 (-0.81, 0.69)	-0.12 (-0.91, 0.40)	-	-0.03 (-0.80, 0.70)	0.36 (-0.32, 0.93)	0.02 (-0.77, 0.73)
Sperm velocity	-0.18 (-0.89, 0.48)	-0.26 (-0.92, 0.34)	-0.08 (-0.86, 0.58)	-0.03 (-0.80, 0.70)	-	-0.08 (-0.76, 0.75)	0.00 (-0.79, 0.71)
Growth	-0.21 (-0.93, 0.27)	-0.40 (-0.96, 0.07)	-0.32 (-0.95, 0.10)	0.36 (-0.32, 0.93)	-0.08 (-0.76, 0.75)	-	-0.02 (-0.79, 0.71)
rTL	-0.04 (-0.83, 0.64)	0.25 (-0.64, 0.83)	-0.18 (-0.89, 0.50)	0.02 (-0.77, 0.73)	0.00 (-0.79, 0.71)	-0.02 (-0.79, 0.71)	-

Table S3. Spearman's correlation coefficients with confidence intervals (in brackets) for each of the traits we measured for winning males that had free access to mating opportunities. Correlations were calculated for complete observations only (where males had all traits measured). Significant correlations indicated with asterisks (*).

Trait	*Mating attempts*	*Successful attempts*	*Time with female*	*Sperm count*	*Sperm velocity*	*Growth*	*rTL*
Mating attempts	-	0.32 (-0.66, 0.82)	0.45 (-0.48, 0.89)	0.10 (-0.87, 0.57)	0.49 (-0.48, 0.89)	0.50 (-0.44, 0.90)	0.03 (-0.92, 0.35)
Successful attempts	0.32 (-0.66, 0.82)	-	0.38 (-0.31, 0.93)	-0.09 (-0.92, 0.33)	0.06 (-0.89, 0.49)	-0.15 (-0.93, 0.26)	-0.01 (-0.87, 0.55)
Time with female	0.45 (-0.48, 0.89)	0.38 (-0.31, 0.93)	-	-0.18 (-0.95, 0.10)	0.01 (-0.89, 0.50)	0.02 (-0.87, 0.55)	-0.02 (-0.88, 0.53)
Sperm count	0.10 (-0.87, 0.57)	-0.09 (-0.92, 0.33)	-0.18 (-0.95, 0.10)	-	0.32 (-0.55, 0.87)	0.16 (-0.69, 0.81)	0.08 (-0.77, 0.73)
Sperm velocity	0.49 (-0.48, 0.89)	0.06 (-0.89, 0.49)	0.01 (-0.89, 0.50)	0.32 (-0.55, 0.87)	-	0.53 (-0.11, 0.95)	0.04 (-0.87, 0.56)
Growth	0.50 (-0.44, 0.90)	-0.15 (-0.93, 0.26)	0.02 (-0.87, 0.55)	0.16 (-0.69, 0.81)	0.53 (-0.11, 0.95)	-	0.10 (-0.81, 0.68)
rTL	0.03 (-0.92, 0.35)	-0.01 (-0.87, 0.55)	-0.02 (-0.88, 0.53)	0.08 (-0.77, 0.73)	0.04 (-0.87, 0.56)	0.10 (-0.81, 0.68)	-

Table S4. Spearman's correlation coefficients with confidence intervals (in brackets) for each of the traits we measured for losing males that had no access to mating opportunities. Correlations were calculated for complete observations only (where males had all traits measured). Significant correlations indicated with asterisks (*).

Trait	*Mating attempts*	*Successful attempts*	*Time with female*	*Sperm count*	*Sperm velocity*	*Growth*	*rTL*
Mating attempts	-	0.39 (-0.32, 0.93)	0.81** (0.52, 0.99)	0.07 (-0.79, 0.72)	-0.35 (-0.94, 0.22)	0.06 (-0.73, 0.77)	-0.27 (-0.92, 0.35)
Successful attempts	0.39 (-0.32, 0.93)	-	0.33 (-0.51, 0.88)	0.08 (-0.82, 0.66)	0.04 (-0.84, 0.64)	0.20 (-0.61, 0.85)	-0.38* (-0.98, -0.24)
Time with female	0.81** (0.52, 0.99)	0.33 (-0.51, 0.88)	-	0.21 (-0.72, 0.79)	-0.04 (-0.89, 0.48)	-0.10 (-0.86, 0.59)	0.03 (-0.86, 0.57)
Sperm count	0.07 (-0.79, 0.72)	0.08 (-0.82, 0.66)	0.21 (-0.72, 0.79)	-	0.12 (-0.67, 0.81)	-0.22 (-0.93, 0.26)	0.15 (-0.64, 0.83)
Sperm velocity	-0.35 (-0.94, 0.22)	0.04 (-0.84, 0.64)	-0.04 (-0.89, 0.48)	0.12 (-0.69, 0.81)	-	-0.22 (-0.90, 0.46)	0.01 (-0.71, 0.79)
Growth	0.06 (-0.73, 0.77)	0.20 (-0.61, 0.85)	-0.10 (-0.86, 0.59)	-0.22 (-0.93, 0.26)	-0.22 (-0.90, 0.46)	-	-0.14 (-0.88, 0.52)
rTL	-0.27 (-0.92, 0.35)	-0.38* (-0.98, -0.24)	0.03 (-0.86, 0.57)	0.15 (-0.64, 0.83)	0.01 (-0.71, 0.79)	-0.14 (-0.88, 0.52)	-

Table S5. Spearman's correlation coefficients with confidence intervals (in brackets) for each of the traits we measured for losing males that had free access to mating opportunities. Correlations were calculated for complete observations only (where males had all traits measured). Significant correlations indicated with asterisks (*).

Trait	*Mating attempts*	*Successful attempts*	*Time with female*	*Sperm count*	*Sperm velocity*	*Growth*	*rTL*
Mating attempts	-	0.33 (-0.35, 0.92)	0.56* (0.08, 0.97)	0.17 (-0.61, 0.85)	0.07 (-0.69, 0.81)	0.14 (-0.74, 0.76)	-0.38 (-0.96, 0.03)
Successful attempts	0.33 (-0.35, 0.92)	-	0.33 (-0.36, 0.92)	0.07 (-0.73, 0.78)	0.23 (-0.50, 0.89)	0.06 (-0.79, 0.70)	-0.40* (-0.96, -0.02)
Time with female	0.56* (0.08, 0.97)	0.33 (-0.36, 0.92)	-	0.43 (-0.34, 0.92)	0.24 (-0.58, 0.86)	0.12 (-0.77, 0.73)	-0.42* (-0.97, -0.21)
Sperm count	0.17 (-0.61, 0.85)	0.07 (-0.73, 0.78)	0.43 (-0.34, 0.92)	-	-0.02 (-0.79, 0.70)	0.10 (-0.77, 0.74)	-0.20 (-0.89, 0.49)
Sperm velocity	0.07 (-0.69, 0.81)	0.23 (-0.50, 0.89)	0.24 (-0.58, 0.86)	-0.02 (-0.79, 0.70)	-	0.05 (-0.79, 0.71)	-0.35 (-0.94, 0.26)
Growth	0.14 (-0.74, 0.76)	0.06 (-0.79, 0.70)	0.12 (-0.77, 0.73)	0.10 (-0.77, 0.74)	0.05 (-0.79, 0.71)	-	-0.04 (-0.78, 0.72)
rTL	-0.38 (-0.96, 0.03)	-0.40* (-0.96, -0.02)	-0.42* (-0.97, -0.21)	-0.20 (-0.89, 0.49)	-0.35 (-0.93, 0.26)	-0.04 (-0.78, 0.72)	-

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Shao, F., Ludwig, A., Mao, Y., Liu, N., & Peng, Z. (2020). Chromosome-level genome assembly of the female western mosquitofish (*Gambusia affinis*). GigaScience, 9(8), giaa092.