The effect of brief or prolonged bouts of winning or losing male-male contests on plasticity in sexually selected traits

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Abstract

The outcomes of fights often affect the fitness of males by determining their access to mates. 'Winner-loser' effects, where winners often win their next contest, but losers tend to lose, can therefore influence how males allocate resources towards traits under pre- and post-copulatory sexual selection. We experimentally manipulated the winning/losing experiences of pairs of size-matched male *Gambusia holbrooki* for either a day, a week or three weeks to test whether prior winning/losing experiences differentially affect the plasticity of male investment into either mating effort (pre-copulatory) or ejaculates (post-copulatory). When winner/loser pairs could directly compete for a female, winners had better pre-copulatory outcomes than losers for three of the four traits we measured: number of mating attempts, number of successful attempts, and time spent with the female (but not aggression). Winners also produced faster sperm than losers, but there was no difference in total sperm counts. Interestingly, absolute male size, an important predictor of fighting success, mediated the effect of winning or losing on how long males then spent near a female. Compared to losers, smaller winners spent more time with the female than did larger winners, suggesting that how males respond to prior social experiences is size-dependent. We discuss the general importance of controlling for inherent male condition when comparing male investment into condition-dependent traits.

Introduction

Male-male sexual competition is often a major determinant of male fitness. Competition among males for access to mates generates pre-copulatory sexual selection that favours traits that increase mating effort like weapons and courtship displays (Darwin 1871; Andersson 1994; Hardy and Briffa 2013). If females mate multiply, males face the additional challenge that their sperm compete to fertilise eggs (Parker 1970; Parker and Pizzari 2010). Post-copulatory sexual selection (e.g. sperm competition) favours male traits that increase sperm competitiveness such as larger testes (Lüpold et al. 2020), bigger ejaculates (Kelly and Jennions 2011), and higher quality sperm (e.g. Boschetto et al. 2011). A key question that arises is how males should optimally allocate their resources between the two types of traits.

Many sexually selected traits are condition-dependent (Rowe and Houle 1996; Macartney et al. 2019), and investment into both mate acquisition and ejaculates is limited by a male's ability to acquire the relevant resources. The total resources available to a male are therefore likely to determine his optimal investment strategy into sexually selected traits (Simmons et al. 2017). For example, males with sufficient resources to be good fighters (i.e. superior armaments or bigger bodies) are more likely to mate, and might benefit relatively less from investing in sperm competitiveness than do males that are poor fighters that obtain matings via other means (e.g. sneak mating; Parker et al. 2013). Alternative reproductive tactics (ARTs; Taborsky et al. 2008) are an extreme form of divergence in reproductive strategies whereby subordinate males that are unlikely to win fights for females, often because they are smaller bodied, are predicted to invest more into traits under post-copulatory sexual selection, like sperm production (i.e. 'sneaker' males). 'Sneaker' males are always subjected to strong sperm competition, but greater investment in ejaculates lowers investment into traits that increase mating success (e.g. weapons; Simmons et al. 2017; but see Kustra and Alonzo 2020). This trade-off is, however, likely to vary across environments or social settings. For example, when intense male-male competition prevents males from monopolising females, higher levels of multiple mating by females shifts male allocation strategies from mating effort towards increased sperm competitiveness (Parker et al. 2013; Lüpold et al. 2014).

When the environmental or social conditions that males experience vary, the relative costs and benefits of allocation to traits under pre- and post-copulatory sexual selection can change. Such interactions between external factors and a male's state can favour phenotypic plasticity in male reproductive strategies for resource allocation (Bretman et al. 2011; Dore et al. 2018, 2020; Fox et al. 2019*a*). For instance, dominant males are predicted to invest more heavily than subordinates into acquiring mating opportunities (Parker et al. 2013). Indeed, many studies report measurable differences between dominant and subordinate males in both pre- and post-copulatory sexual traits (e.g. Montrose et al. 2008; Simmons and Buzatto 2014; Reuland et al. 2021). There is also evidence that these differences reflect plastic responses to changes in social ranking. For example, observational studies showed that dominant male domestic fowl (*Gallus gallus domesticus*) produce more sperm, but its quality decreases faster over successive copulation attempts than that of subordinate males (Cornwallis and Birkhead 2007). But when the social status of dominant and subordinate males was experimentally switched, previously dominant males then produced fewer sperm, but of high quality, over successive copulations (Cornwallis and Birkhead 2007). In nature, the relative status of a male within a social hierarchy is usually underpinned by differences in body size or condition. This begs the question of how the outcome of contests between otherwise equally matched males affects their reproductive investment strategies. Does winning such an encounter increase the likelihood of winning again in the future? And, if so, how does this affect the optimal allocation of resources?

Animal contests are primarily decided by asymmetries in physical traits (e.g. weapon or body size; Jennions and Backwell 1996), persistence (e.g. the initiation and escalation of aggressive interactions; Kar et al. 2016), or even luck. The outcome of prior competitive interactions can also have long-lasting effects on contestants when they face new rivals; winners are more likely to behave aggressively and win future fights, and losers are less likely to escalate fights and tend to lose (Hsu and Wolf 2001). Such 'winner-loser' effects persist even when intrinsic differences in fighting ability or motivation (i.e. resource holding potential, RHP: Parker 1974; Arnott and Elwood 2008) are absent. That is, winners and losers have different carry-over effects influencing their future fighting behaviour even when there is no difference between them in key predictors of fighting ability. In nature, the ability of males to assess their own and their rival's fighting ability (see contest strategies reviewed by Chapin et al. 2019) means that many fights are between closely matched individuals. This, in turn, means that luck or other extrinsic factors will often determine contest outcomes. Winner-loser effects can therefore play an important role in male-male competition for females because carry-over effects from prior contests are likely to affect male fitness. Indeed, males that consistently win fights often have greater access to resources, like territories (Kemp and Wiklund 2004) and mates (Bierbach et al. 2013), and are more motivated to fight rivals (Bergman et al. 2010).

Past winners of fights can benefit from increased future mating opportunities. For example, winning males generally invest more into courtship behaviours that attract females. Winning male *Velarifictorus aspersus* crickets increase their call rate (Zeng et al. 2018); and male tilapia (*Oreochromis mossambicus*) that win fights produce more courtship sounds, and for longer periods of time, than do losers (Amorim and Almada 2005). Similar differences in traits under post-copulatory sexual selection also arise. For example, after losing a fight, male broad-horned flour beetles (*Gnatocerus cornutus*) transfer more sperm during mating (Okada et al. 2010), while losing male crickets (*Gryllus bimaculatus)* produce higher quality sperm (Tuni et al. 2016). These intriguing findings, after a single contest, suggest that losers might switch to greater investment into traits under post-copulatory sexual selection. However, these studies involved males that naturally won or lost fights, so it is possible that losers were generally inferior to winners (i.e. selection bias in sampling winners and losers; see Hsu et al. 2006), which already caused winners to invest relatively more into mating and losers into sperm traits (as with ARTs, see above). For example, in a subsequent study of *G. bimaculatus* crickets where winner-loser roles were experimentally created, the sperm quality of winners and losers did not differ (Tuni et al. 2019). When testing how fight outcomes affect male investment strategies it is therefore prudent to randomly assign males to either win or lose fights to control for any intrinsic differences in their quality or condition (e.g. Harrison et al. 2018).

While winner-loser effects have dramatic effects on some facets of male reproductive success, winners do not necessarily have greater fitness (e.g. Zeng et al. 2018). This is possible if winning or losing males shift their investment among traits to compensate fully for a lower rate of return from investment in certain traits, especially where these traits affect only some of the events that determine net reproductive success (Parker et al. 2013). For example, Filice and Dukas (2019) found that winning male *Drosophila melanogaster* flies have higher mating success than losing males. However, losers mated for longer and sired more offspring when they were the first to mate with a female, suggesting that losers invested more into traits under post-copulatory sexual selection (Filice and Dukas 2019). The difference in investment in reproductive strategies by winners and losers yielded the same fitness outcome. It is therefore necessary to test for winner-loser effects on traits that affect mating success and those that affect sperm competitiveness. Many studies only examine one component of male reproductive success.

Winner-loser effects involve physiological changes in response to the immediate environment (i.e. the experience of winning or losing: Hsu et al. 2006; Earley and Hsu 2008; Earley et al. 2013) that are assumed to reflect adaptive phenotypic plasticity. The duration of a winning or losing experience should, however, provide additional information about a male's likely future success, hence the relative gains from further shifts in investment into traits under pre- and post-copulatory sexual selection. The more extensive the experience of winning or losing the greater the likelihood of a shift in male investment. For example, we might expect repeated losers to reduce their mating effort and increase their investment in sperm competitiveness. To date, few studies have experimentally manipulated the contest experience of males to determine how it affects investment into traits under pre- and post-copulatory sexual selection (but see Filice and Dukas 2019; Tuni et al. 2019).

Here, we fill key gaps in our understanding of how a male's social environment, specifically his dominance status (controlling for inherent variation in male quality) affect adaptive plasticity in male reproductive strategies. To do this we experimentally manipulated the contest experience of male *Gambusia holbrooki* mosquitofish to create size-matched winners and losers. Mosquitofish are a good study system to test the plasticity of male investment in reproduction in response to winning or losing. Males spend a large proportion of their time fighting and harassing females to mate. Males thus face strong pre- and post-copulatory sexual selection. In addition, earlier work on this species found that, when size-matched winners and losers competed directly, prior winners increase their association times with females (Harrison et al. 2018). In the present study we made males consistently experience bouts of winning or losing for either a day, a week, or three weeks, to test if winning affects: a) how males allocate resources to traits under either pre- or post-copulatory sexual selection, b) if any plasticity in allocation changes with the duration of a male's contest experience, and c) whether absolute male body size, a trait itself under sexual selection, mediates plastic shifts in the allocation of investment.

Experimental Methodology

Study species

Gambusia holbrooki are a promiscuous poecilid species that naturally form high density mixed-sex shoals. Male mosquitofish are aggressive towards each other, and larger males are socially dominant (Caldwell and Caldwell 1962; McPeek 1992). Contests between similarly-sized males begin with lateral displays where males circle each other that then escalate to fin nipping and end with the winner chasing the loser (McPeek 1992). Winners spend more time associating with females than losers (i.e. guarding her from other males; see Harrison et al. 2018) which suggests that, over time, winners should have higher fitness than losers. Because males and females mate multiply, there is intense male-male competition for mating opportunities and sperm competition to fertilise eggs (e.g. Zane et al. 1999). Males harass females and force copulations by swinging their gonopodium (modified anal fin) forwards before thrusting it towards her gonopore (Bisazza and Marin 1995). Although females tend to prefer to associate with larger males (Aich et al. 2021), this does not appear to consistently elevate the mating success of large males (e.g. Pilastro et al. 1997; Booksmythe et al. 2013; Head et al. 2015).

Animal collection and maintenance

Mature adult mosquitofish (identified by a hook-like tip to the gonopodia of males and a gravid spot in females) were wild-caught in Canberra (35°14'30.1"S 149°06'17.0"E) during summer 2020-21 (Dec-Feb). Fish were brought back to aquarium facilities at The Australian National University and housed in same-sex stock aquaria (90 L; ~50 individuals/aquarium) at 28 ± 1°C under a 14 L:10 D hour photoperiod. Stock fish were fed *ad libitum* with commercial fish flakes, and experimental fish were given *Artemia salina* nauplii *ad libitum* twice daily. All animal collection and experimental work was conducted under ethics protocol A2021/04.

Experimental design: making winners and losers

To investigate how prior winning or losing experiences affect male reproductive investment, we experimentally manipulated the contest experiences (winning or losing) of males for either 1 day, 1 week, or 3 weeks. Winners were made to face smaller rivals while losers faced larger rivals continuously throughout their contest duration period. We then measured a set of key traits on focal males that are under pre- or post-copulatory sexual selection. We randomly selected focal males from the stock population to test for winner-loser effects in males of all sizes to extend the findings of Harrison et al. (2018). Our earlier study used a smaller size range of relatively large males (size range: 22-27 mm) and observed winner-loser pre-copulatory interactions for only 10 mins. We assigned the very smallest (<18 mm) and largest (>25 mm) males in our stock population as rival competitors. One week prior to experimental treatments, focal males (N = 516) were anaesthetised briefly in an ice slurry for 30 secs to measure their standard body length (SL) with dial callipers and to tag them with a subcutaneous elastomer tag (NorthWest Marine Technology, Washington, USA) for identification (a process that takes only 30 secs). Focal male SL ranged from 17.4 – 26.9 mm (mean ± SD: 21.02 ± 1.81 mm). Based on visual assessment, size differences between focal and competitor males were usually ~3 mm but likely ranged between 1-7 mm. Males were then kept in individual 1 L tanks for one week prior to competitive trials.

We experimentally created winners and losers by randomly assigning size-matched focal male pairs to compete against either a smaller (winners) or larger (losers) competitor male (see Harrison et al. 2018; Figure 1). Size differences are an important determinant of social dominance in mosquitofish (Caldwell and Caldwell 1962). By randomly assigning focal males to become winners or losers we could eliminate intrinsic differences in RHP (Parker 1974; Arnott and Elwood 2008) between winners and losers. Focal males either won or lost contests for 1 day, 1 week or 3 weeks (Figure 1). Winning/losing experiences were staggered such that each contest experience treatment ended on the same day for a given block of males ($N = 6$ treatment groups). Contest experiences were broken up into 20 blocks to measure pre-copulatory investment and 21 blocks to measure post-copulatory investment. Each block had two sets of the three winner/loser duration treatments ($N = 12$ pairs per block). In each winning/losing trial a focal and a competitor male interacted freely in a 6 L aquarium with a stimulus female (randomly chosen from the stock population) present behind a mesh barrier to encourage agonistic interactions but prevent mating (Spagopoulou et al. 2020). Contest aquaria contained gravel, plastic plants and were lined with black plastic to minimise outside disturbance. Competitor males were rotated every ~3 days to ensure that focal males were continually winning/losing contests, while stimulus females were rotated every seven days to keep males motivated to fight (Vega-Trejo et al. 2014). At the end of their contest experience winners and losers from the same contest duration treatment were randomly assigned to either compete directly for a female to measure pre-copulatory investment or to have their sperm traits measured (post-copulatory investment) (Figure 1).

Mortality

Contest treatments ended with fewer than the intended 40 males for each of the six winner/loser by contest duration combinations due to natural mortality. Twelve of the 516 males (~2% of our total sample size) died during the first isolation period post-tagging prior to being allocated to an experimental group. Of the remaining 504 males, 39 died during their experimental contest experience. There was significantly higher mortality for losers than winners ($\chi^2 = 4.33$, df = 1, $P = 0.037$) and mortality also differed between the three contest duration treatments ($\chi^2 = 9.69$, df = 2, $P = 0.008$). It was highest for the three-week treatment. Although we expected total mortality to increase with treatment duration, the significant difference in mortality between winners and losers might indicate a weak selection bias towards losers that could better survive in a challenging competitive environment.

Pre-copulatory investment

To compare male investment into pre-copulatory mating behaviours, we placed size-matched (paired *t*-test: mean difference = 0.01 mm, $t = 0.54$, df = 105, $P = 0.594$) focal male pairs (winner and loser from the same contest duration treatment; $N = 106$ dyads) together in a new, 6 L aquarium with a stock female. All females were only used once. Male interactions were observed for 20 mins where we recorded: a) time spent near the female, b) number of mating attempts, c) number of successful mating attempts, and d) aggression directed towards the rival. Mating attempts were recorded each time a male swung his gonopodium forwards towards the female's gonopore. These mating attempts are unambiguous and easy to quantify. Successful mating attempts were recorded when the gonopodium touched the gonopore, potentially transferring spermatophores. Successful mating attempts involve the male twisting his body and the female attempting to roll away from him. We used stopwatches to record the time each male spent within ~5 cm of the female (interacting with or guarding her from rival approaches). Finally, aggression was recorded as how often the male displayed aggressively, nipped, or chased his rival. In total we measured the behaviours of 36 pairs of 1-day treatment males, 38 pairs of 1-week treatment males and 32 pairs of 3-week contest treatment males.

Post-copulatory investment

To compare male investment into post-copulatory traits, focal males ($N = 248$) were isolated and stripped of their sperm to determine how their sperm reserves were affected by winning or losing. They were then stripped again seven days later to measure the effect of winning/losing on rates of sperm replenishment or sperm traits. Sperm collected immediately post-treatment provided baseline measures of the number and velocity of sperm produced by males prior to or during the contest treatment, while replenished sperm are presumably directly influenced by the male's contest experience. As such, we expected a quantifiable difference between the two measures. We measured three key indicators of ejaculate quality: sperm count, sperm velocity (swimming speed) and sperm replenishment rates (comparing current and baseline counts).

a) Sperm collection

At the end of their contest experiences, focal males were anaesthetised briefly in ice slurry and sperm bundles were then stripped by gently massaging the ventral area directly above the base of the gonopodium (see O'Dea et al. 2014). This process removes most sperm (Vega-Trejo et al. 2016), while a seven-day period thereafter allows males enough time to replenish sperm reserves to measure sperm replenishment rates (O'Dea et al. 2014). Two samples of three sperm bundles each were collected and set aside for sperm velocity analysis. The remaining bundles were pipetted into an Eppendorf tube containing 100-1100 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)) to count sperm. Sperm collection and subsequent trait measurements were performed blind to male contest treatment.

b) Sperm count

To estimate total sperm count we vortexed the sperm sample for ~1 min and then repeatedly pipetted the solution (10-20 times) to break up sperm bundles and disperse sperm throughout the sample. We pipetted 3 µL of the mixed sperm solution onto a 20-micron capillary slide (Leja) and counted sperm using a CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA) under x100 magnification. Threshold values defining cell detection were predetermined as elongation percentage 15-65 and head size 5-15 µm (static tail filter set off see: Vega-Trejo et al. 2019; Chung et al. 2021). For sperm counts, we randomly counted five subsamples per sample and used the average. The repeatability of our count subsamples for each male was obtained using the R package *rpt*R (Stoffel et al. 2017). Sperm subsample counts for each male were highly repeatable on both Day 0 (R = 0.90; 95% CIs: 0.88, 0.93; P <0.001) and

Day 7 (R = 0.84; 95% CIs: 0.81, 0.87; $P < 0.001$). We then obtained the total sperm counts by adding the average sperm number per bundle for the six bundles removed for sperm velocity analyses. We measured the total sperm count of 205 males on Day 0 (baseline) and 220 males on Day 7 post-treatment (replenished); hereafter referred to as old and new sperm, respectively.

c) Sperm velocity

To measure sperm velocity, we used two samples from each male's ejaculate (3 sperm bundles each in 3 μL of extender medium). We then 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) to prevent sperm from sticking to the slide. Each sample was then 'activated' with 3 μL of activator solution (125 mM KCL and 2 mg/mL bovine serum albumin) to mimic the chemical environment of the reproductive tract of female *G. holbrooki* and covered with a coverslip. We recorded two standard measures of sperm velocity – VAP (average path velocity) and VCL (curvilinear velocity) using a CEROS Sperm Tracker. Threshold values for defining static cells was predetermined at 20 μm/s for VAP and 15 μm/s for VCL (Gasparini et al. 2010, 2013; Chung et al. 2021). Our measures of VAP and VCL were highly correlated (Pearson's $r = 0.99$, $N = 372$, $P < 0.001$). We used VCL for our analysis because it is a more biologically relevant measure (Vega-Trejo et al. 2019). Sperm velocity measures were obtained from 182 males for old sperm and 190 males for new sperm.

Statistical analyses

a) Pre-copulatory investment

We fitted generalised linear mixed models (GLMMs) with negative binomial error distributions (log-link function) to test for an interaction between winning/losing contest experiences and contest duration on the four key male pre-copulatory traits: the number of mating attempts made, the number of successful mating attempts made, the time each male spent with the female and the absolute rate of aggression towards the rival male. For our full models, each of the four traits were set as the response variable in separate models. In each model, contest experience, contest duration, and their interaction, were treated as fixed categorical factors. Pair ID (winner and loser pair ID) and block ID were random effects. Where the interaction term was not significant, it was removed from the model to quantify main effects. We present the reduced, main effects only models in Table 1.

b) Post-copulatory investment

For post-copulatory investment, we first fit separate GLMMs for each response variable with Gaussian error distributions (identity-link function) to test for any two-way interactions between contest experience, contest duration and sperm age (old vs new sperm). These models had sperm count (log-transformed) and sperm velocity (VCL) as the response variable and contest experience, contest duration, sperm age and male body size, and all two-way interactions, as fixed factors. Male body size was included as a fixed covariate in these models as we had an *a priori* expectation that male size and sperm traits would be positively correlated (O'Dea et al. 2014). Block ID was a random effect. Male ID was included as a random effect to account for two sperm measures per male (i.e. old and new sperm). Next, we fit a second set of GLMMs (Gaussian error with identity-link function) for only the new sperm (replenished). We chose to run models on replenished sperm because there was a significant interaction between sperm age and contest experience (see Results). These models included contest experience, contest duration and male body size, and all two-way interactions, as fixed factors. Only block ID was included as a random effect. Where interaction terms were not significant, they were removed from the final, reduced model to quantify main effects. We present the reduced models with main effects and significant interaction terms in Table 2.

c) Pre-copulatory traits and their interactions with body size

We had an *a priori* expectation that male and female body size might influence how the experimental treatments affected pre-copulatory mating behaviour (e.g. Harrison et al. 2018), but our main focus was on maximising our power to test for the effects of our chosen experimental treatments (i.e. the duration of winning/losing). We therefore ran additional models that are exploratory in nature. Specifically, we again fit GLMMs (negative binomial error with log-link function) for each of the four pre-copulatory traits. These models had contest experience, contest duration, and their interaction, as fixed categorical factors, and also included male and female body size (both centred and standardised to the mean) and their interaction. Pair ID and block ID were again set as random effects. Model parameter estimates are presented in Table 3.

All analyses were conducted using R version 4.0.2 (R Development Core Team 2020). We used the package *glmmTMB* (Brooks et al. 2017) to first 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 (see Supplementary Material for model fitting). We removed all non-significant interactions and used log-likelihood ratio tests to compare model fit. We left main effects in our reduced models (see model descriptions above) because our study was explicitly designed to test if they would affect male investment. We used the *DHARMa* package (Hartig 2020) to run model diagnostics. To obtain the significance of each of the fixed effects we used ANOVA type II Wald chi-square (χ^2) tests in the reduced models and type III in the models that include interaction terms. We set $\alpha = 0.05$ for all model terms except three-way interaction terms (where α was 0.01). All tests were two-tailed. We provide the raw data and R code used for analysis and data visualisation as Supplementary Material.

Results

Pre-copulatory investment

Surprisingly, we found no significant interactions between contest experience and prior contest duration for any of the four pre-copulatory traits we measured, suggesting that plasticity in allocation towards pre-copulatory mating behaviours does not change with the duration of a male's contest experience (Table 1). Winners and losers differed for three of the four traits that we measured (Figure 2). When winners and losers directly competed for a female, winners made significantly more mating attempts ($\chi^2 = 4.90$, df = 1, $P = 0.027$; Figure 2A), more often made successful mating attempts ($\chi^2 = 5.63$, df = 1, $P = 0.018$; Figure 2B), and spent more time near the female ($\chi^2 = 19.62$, df = 1, $P < 0.0001$; Figure 2C) than losers. However, winners and losers did not differ significantly in how many aggressive interactions they initiated ($\chi^2 = 2.25$, df = 1, $P = 0.134$; Figure 2D).

Prior contest experience duration had no significant effect on the number of mating attempts ($\chi^2 = 1.76$, df = 2, $P = 0.414$) nor the time spent near the female ($\chi^2 = 1.43$, df = 2, $P = 0.488$). However, the number of successful attempts made ($\chi^2 = 9.45$, df = 2, $P = 0.009$), and male aggression towards each other ($\chi^2 = 9.92$, df = 2, $P = 0.007$), increased significantly with the duration of their prior contest experience for both winners and losers. After 3 weeks of contests, both winners and losers more often made successful mating attempts, and were more aggressive, than males that had experienced only 1 day or 1 week of contests (Table 1).

Post-copulatory investment

When considering both old and new sperm, there were no significant interactions between contest experience and prior contest duration for either sperm counts or sperm velocity (Table 2). Winning/losing experiences also had no significant effect on either sperm count (winning: $\chi^2 = 0.80$, df = 1, $P = 0.371$; Figure 3A) or sperm velocity ($\chi^2 = 2.62$, df = 1, $P = 0.106$; Figure 3B). For sperm counts, there was, however, a significant interaction between sperm age and the duration of the prior contest experience ($\chi^2 = 7.87$, df = 2, $P = 0.020$). Males that experienced only 1 day of contests replenished their sperm stores to baseline levels, while males that experienced either 1 week or 3 weeks of contests did not (Table 2). New, replenished sperm was significantly faster than old sperm ($\chi^2 = 4.15$, df = 1, $P = 0.042$), but there was no effect of contest treatment duration on sperm velocity ($\chi^2 = 2.60$, df = 2, $P = 0.272$; Table 2).

When only replenished sperm were examined, larger males produced significantly more sperm ($\chi^2 = 11.16$, df = 1, $P = 0.0008$), but there was no effect of winning or losing ($\chi^2 = 0.66$, df = 1, $P = 0.417$) nor of contest duration ($\chi^2 = 3.23$, df = 2, $P = 0.200$) (Table 2). Winners replenished significantly faster sperm than losers ($\chi^2 = 4.34$, df = 1, $P = 0.037$), but there was no effect on replenished sperm velocity of either male body size ($\chi^2 = 0.00$, df = 1, $P = 0.951$) or contest treatment duration ($\chi^2 = 0.86$, df = 2, $P = 0.652$) (Table 2).

Pre-copulatory traits and their interactions with body size

Compared to males that had only 1 day of contest experience, males that experienced 1 or 3 weeks of contests made significantly fewer mating attempts towards larger females ($\chi^2 = 8.97$, df = 2, $P = 0.011$; Figure 4A). In addition, smaller males more often made successful mating attempts than did larger males ($\chi^2 = 7.88$, df = 1, $P = 0.005$; Figure 4B).

Interestingly, there was a significant three-way interaction between contest treatment, contest duration and male body size that affected how long a male spent near the female ($\chi^2 = 8.80$, df = 2, $P = 0.012$; Table 3). How a male responded to his contest experience and its duration was moderated by his body size (Figure 4C). Smaller winners tended to spend more time than larger winners associating with the female, especially after 1 or 3 weeks of contests. While after 1 week of contests, larger losers spent more time than smaller losers near females. Neither male nor female body size affected male aggression (male size: $\chi^2 = 0.22$, df = 1, $P = 0.639$; female size: $\chi^2 = 0.49$, df = 1, $P = 0.482$; Table 3).

Discussion

Since the outcome of male-male contests can determine access to both females and resources, it is plausible that winners and losers plastically adjust their investment into condition-dependent sexually selected traits. To test this, we manipulated the extent to which male mosquitofish, *Gambusia holbrooki*, had a recent history of winning or losing contests. We predicted that winners would subsequently have higher mating success than losers, and that losers would therefore benefit from investing more into sperm traits that are under post-copulatory sexual selection. Winners did indeed have greater pre-copulatory success than losers for three of the four behavioural traits we measured (the exception being aggression). This finding of a strong winner effect on male mating effort both corroborates and extends earlier work on *G. holbrooki* (Harrison et al. 2018). Contrary to predictions, however, winners invested more than losers into an ejaculate trait that is likely to be under post-copulatory sexual selection: winners produced significantly faster sperm than losers, although there was no effect of winning or losing on sperm count. This finding is surprising as males with poor fighting abilities are widely predicted to produce larger amounts and/or more competitive sperm to increase their success under sperm competition (Parker 1990; Parker et al. 2013). Interestingly, the magnitude of these plastic responses to winning or losing contests was unaffected by the duration of their earlier contest experience. However, absolute male size, which predicts social dominance in *G. holbrooki* (Caldwell and Caldwell 1962), moderated the plastic response to winning or losing contests for the time that males then spent near the female, although this sometimes depended on the duration of the contest experience. Our findings highlight the importance of experimentally controlling for intrinsic differences between males (e.g. body size) when investigating the plasticity of investment into condition-dependent sexual traits in response to the experience of winning (but not losing) past encounters.

Reproductive allocation trade-offs

When males and females mate multiply, males should trade-off investment into traits under pre- or post-copulatory sexual selection to favour whichever provides greater marginal fitness gains (Parker et al. 2013; Simmons et al. 2017). Evidence of such trade-offs is mainly limited to studies that compare males that vary in body condition (De Nardo et al. 2021), dominant versus subordinate males (Gage et al. 1995; Warner et al. 1995), or males using alternative reproductive tactics (Simmons and Buzatto 2014). These studies provide limited scope to interpret adaptive plasticity in response to winning or losing fights because contest outcome is often determined by inherent male quality or body condition which, as shown in our study, also affects investment into sexually selected traits. A similar problem emerges when asking if winning or losing natural fights affects a male's subsequent mating effort (e.g. Okada et al. 2010; Tuni et al. 2016; Zeng et al. 2018). In our study, we experimentally created winners and losers using size-matched male *G. holbrooki* and then allowed these males to compete. Males had a consistent winning or losing contest experience for either a day, a week or three weeks before we measured putative sexually selected traits (Bisazza and Marin 1995; O'Dea et al. 2014; Fox et al. 2019*b*). Although there was a difference between winners and losers in behaviours that affect mating success, losers did not appear to reallocate resources to traits under post-copulatory sexual selection. This was unexpected because this is one way in which losers can potentially compensate for their reduced mating effort, hence likely lower mating success.

A key question that arises is why don't males adjust their allocation strategies in response to winning or losing when contest outcomes can affect both their future body condition and mating success? There are several potential explanations. First, life-history strategies might constrain the benefits males gain from reallocating resources towards different aspects of reproduction. *Gambusia holbrooki* males only survive for one breeding season (Kahn et al. 2013). As such, plastic adjustments in reproductive allocation by males in *G. holbrooki* and other shortlived species can only affect fitness in the current breeding season (e.g. semelparous marsupials; Fisher et al. 2013). Winning and losing experiences might therefore favour shifts in other life-history traits, such as growth rates or longevity, that have larger effects on fitness than small changes in allocation between sperm competitiveness and mating effort. For instance, male painted dragons (*Ctenophorus pictus*) have a polymorphism with different reproductive strategies. Red-headed males invest relatively more into male-male competition but have shorter telomeres, a proxy for longevity, compared to yellow-headed males that invest into sperm competition (Rollings et al. 2017). Similar trade-offs between reproductive effort and lifespan occur in other species with continuous variation in reproductive tactics (e.g. Lemaître et al. 2015, 2020).

Second, male coercive mating is an 'activational' behaviour (*sensu* Snell-Rood 2013) that can be more rapidly adjusted than most ejaculate traits in response to environmental factors or social cues. In vertebrates, both male mating effort and contest performance are regulated by testosterone (Earley et al. 2013). Based on known physiological mechanisms it is plausible that higher testosterone levels after winning a fight facilitate an immediate plastic response in other behavioural traits, such as mating (Lane and Briffa 2021). There is weaker evidence that winning or losing fights affects ejaculate traits (e.g. Filice and Dukas 2019; Tuni et al. 2019). Although *G. holbrooki* males replenish sperm reserves after ~5 days (O'Dea et al. 2014), the entire spermatogenesis cycle is ~22 days (Koya and Iwase 2004; Schulz et al. 2010). Sperm production is therefore less likely to be adjusted in response to short-term changes in the social environment. In our study, we measure both total available sperm and sperm velocity as indicators of investment into ejaculates. Both are biologically relevant traits (see Chung et al. 2021), but it is unknown how any changes affect paternity when winners and losers compete. For example, in a study of winner-loser effects in *Drosophila melanogaster* flies, losers gained more paternity than winners if they were allowed to mate first (Filice and Dukas 2019). This suggests that losers might use other tactics, such as strategic sperm allocation (Parker and Pizzari 2010), to increase their share of paternity rather than elevate sperm production or produce faster sperm.

Third, if the energetic costs of mating competition or sperm production are low, there might be minimal trade-offs between traits under pre- and post-copulatory sexual selection (i.e. mating effort and ejaculates; Parker et al. 2013). However, if male-male competition is such that males cannot readily monopolise females, all males tend to invest more into ejaculate traits that increase sperm competitiveness because of higher marginal fitness gains (Lüpold et al. 2014). We found that male *G. holbrooki* adjusted both pre- and post-copulatory traits following a winning experience. This suggests that mating effort and sperm performance are both important in determining male reproductive success, but this still begs the question why there was no loser effect. It is worth noting, however, that trade-offs are obscured when looking across individuals if there is high inter-individual variation in resource acquisition (van Noordwijk and De Jong 1986). Winning contests tends to increase access to food, while losing males might spend more energy avoiding agonistic interactions. Both scenarios are likely to have occurred during our experiments. In *G. holbrooki*, sperm production is relatively cheap compared to mating behaviour (Chung et al. 2021). It is possible, then, that winners allocate the additional energetic resources gained from greater access to food towards both pre- and post-copulatory traits so that no trade-off was detected when comparing winners and losers (van Noordwijk and De Jong 1986; De Jong and van Noordwijk 1992).

Plasticity and body size

Plastic shifts in male reproductive allocation in response to social competition have been reported for some promiscuous species (e.g. *D. melanogaster:* Dore et al. 2020), including *G. holbrooki* (Spagopoulou et al. 2020). It is therefore surprising that we did not find plasticity in response to the duration of winning or losing experiences for the traits we measured. One explanation is that the duration of winning or losing is an unreliable cue of the future social environment (Dore et al. 2018). For instance, if males do not keep count of prior wins or losses, or winner effects decay rapidly, then only the most recent contest experience is relevant (Hsu and Wolf 1999; Kasumovic et al. 2010). Another explanation is that the adaptive value of plasticity is partly determined by the cost of its expression (DeWitt et al. 2008). If reproductive plasticity is costly, males might be more sensitive to other cues, such as resource availability (e.g. Dore et al. 2020) or body size (e.g. De Nardo et al. 2021). For example, body size is usually a reliable cue of male competitive ability: small males will have many larger rivals that favour always investing into sperm competitiveness (e.g. the size-based mating tactics of *Poecilia latipinna* sailfin mollies: Travis and Woodward 1989).

Body size often affects fight outcome and determines access to both resources and mates. Male size should therefore play a key role in the allocation of resources towards sexually selected traits because it affects the net benefits of engaging in contests (e.g. Kasumovic et al. 2011; Mitchem et al. 2019). For instance, male-male contests are more intense for hissing cockroaches (*Gromphadorhina portentosa*) of intermediate size because they have more to gain or lose by investing in fighting than do large or small males (Logue et al. 2011). Winner-loser effects on other male traits can be similarly modulated by a male's position within a dominance hierarchy. In our study, we found that for one of the four key traits under pre-copulatory sexual selection (i.e., time spent with the female) smaller males responded more strongly to a winning experience than did larger males, which suggests that the marginal fitness gains are greater for them. This might occur because individuals learn their position within a dominance hierarchy based on past contests, which affects their subsequent interactions (Leimar 2021; Leimar and Bshary 2021). In natural interactions, where body size differences exist, losers are often smaller. Smaller males might learn that they are natural losers (see Taborsky and Oliveira 2012). Winning is therefore a rare experience for a smaller male and might disproportionately affect his response. For example, social cichlids (*Neolamprologus pulcher*) form linear dominance hierarchies based on body size. After a winning experience, dominant individuals were more likely than subordinates to escalate subsequent fights, be aggressive and win subsequent contests (Lerena et al. 2021). In *G. holbrooki*, however, we found that smaller males responded more strongly to winning contests than did larger males. Small males that won staged contests might have done so because their victory provided a social cue that their likelihood of success in future encounters had been elevated. In contrast, victory for a large male simply confirmed that his status was unchanged.

Conclusions

By experimentally manipulating the social experiences of size-matched males to make them consistent winners or losers, we showed that winning-losing experiences have immediate consequences for subsequent male mating effort. However, winner-loser effects did not change how males allocated resources to mating effort versus ejaculates. In addition, we found that male body size had an important role in mediating responses to contest outcomes with respect to the time that males spent associating with a female. This implies that the marginal fitness gain from investment into mating effort and ejaculates partially depends on male body size. Finally, our findings suggest that prior winning experiences, even in the absence of differences in male condition or fighting ability, have important consequences for male allocation towards reproduction.

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Statement of authorship

L.M.H. and M.D.J. conceived the idea for the study. L.M.H. carried out all experimental work and performed the data analysis, data visualisation and interpretation. R.V.-T. and M.D.J. contributed to data interpretation. L.M.H. wrote the first draft and R.V.-T. and M.D.J. provided critical revisions. M.D.J. supervised the project and provided funding. All authors approve the final version submitted for publication.

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Figures and Tables

Table 1. Parameter estimates and summary statistics (Type II ANOVA) from the reduced generalised linear mixed models (negative binomial error distributions, fixed effects only) for the four different pre-copulatory traits. Significant effects are indicated by an asterisk (*).

	Model Output		*Summary Statistics*	
Model parameters	**Model estimate**	**SE**	χ^2 **(df)**	***p*-value**
1. *Mating attempts*				
Intercept (Loser, 1 Day)	2.09	0.32		
Contest treatment (Winner)	0.47	0.21	4.90 (1)	0.03*
Contest duration (1 Week)	0.13	0.32	1.76 (2)	0.41
Contest duration (3 Weeks)	0.43	0.34		
2. *Successful attempts*				
Intercept (Loser, 1 Day)	-0.57	0.26		
Contest treatment (Winner)	0.52	0.22	5.63 (1)	0.02*
Contest duration (1 Week)	-0.13	0.28	9.45 (2)	0.009**
Contest duration (3 Weeks)	0.63	0.27		
3. *Time with female*				
Intercept (Loser, 1 Day)	4.28	0.14		
Contest treatment (Winner)	0.65	0.15	19.62 (1)	<0.0001***
Contest duration (1 Week)	0.05	0.18	1.43 (2)	0.49
Contest duration (3 Weeks)	0.21	0.18		
4. *Aggression towards rival*				
Intercept (Loser, 1 Day)	0.83	0.25		
Contest treatment (Winner)	0.30	0.20	2.25 (1)	0.13
Contest duration (1 Week)	0.74	0.29	9.92 (2)	0.007**
Contest duration (3 Weeks)	0.85	0.30		

Table 2. Parameter estimates and summary statistics ((Type II for fixed effects only models, Type III for models with interactions) from the reduced generalised linear mixed models (Gaussian error distributions) for sperm traits of baseline (old) and replenished (new) sperm and GLMMs for new sperm traits only. Significant effects are indicated by an asterisk (*).

	Model Output		*Summary Statistics*	
Model Parameters	**Model estimate**	**SE**	**χ^2 (df)**	***p*-value**
1. *Sperm count*				
Intercept (Loser, 1 Day, Old sperm)	10.51	1.59	43.86 (1)	<0.0001***
Contest treatment (Winner)	-0.07	0.08	0.80 (1)	0.37
Contest duration (1 Week)	0.46	0.13	18.91 (2)	<0.0001***
Contest duration (3 Weeks)	0.51	0.13		
Sperm age (New)	0.10	0.11	0.76 (1)	0.38
Male body size (standardised)	1.34	0.52	6.60 (1)	0.01*
Contest duration (1 Week) x Sperm age (New)	-0.41	0.15	7.87 (2)	0.02*
Contest duration (3 Weeks) x Sperm age (New)	-0.32	0.15		
2. *Sperm velocity*				
Intercept (Loser, 1 Day, Old sperm)	109.30	67.22		
Contest treatment (Winner)	5.12	3.15	2.62 (1)	0.11
Contest duration (1 Week)	1.67	3.88	2.60 (2)	0.27
Contest duration (3 Weeks)	-4.34	3.86		
Sperm age (New)	6.38	3.13	4.15 (1)	0.04*
Male body size (standardised)	3.96	21.95	0.01 (1)	0.91
3. *Sperm count (new sperm only)*				
Intercept (Loser, 1 Day)	14.68	0.10		
Contest treatment (Winner)	-0.07	0.09	0.66 (1)	0.42
Contest duration (1 Week)	0.04	0.11	3.23 (2)	0.20
Contest duration (3 Weeks)	0.18	0.11		
Male body size (standardised)	0.16	0.05	11.16 (1)	0.0008***
4. *Sperm velocity (new sperm only)*				
Intercept (Loser, 1 Day)	124.38	85.81		
Contest treatment (Winner)	8.30	3.98	4.36 (1)	0.04*
Contest duration (1 Week)	-2.40	4.95	0.86 (2)	0.65
Contest duration (3 Weeks)	-4.51	4.88		
Male body size (standardised)	1.73	28.04	0.03 (1)	0.95

Table 3. Parameter estimates and summary statistics (Type II for fixed effects only models, Type III for models with interactions) from the reduced generalised linear mixed models (negative binomial error distributions, significant interaction terms) for pre-copulatory traits including male and female body size. Significant effects are indicated by an asterisk (*).

	Model Output		*Summary Statistics*	
Model parameters	**Model estimate**	**SE**	χ^2 **(df)**	***p*-value**
1. Mating attempts				
Intercept (Loser, 1 Day)	2.11	0.24	74.93 (1)	<0.0001***
Contest treatment (Winner)	0.40	0.21	3.77 (1)	0.05*
Contest duration (1 Week)	0.20	0.25	3.60 (2)	0.17
Contest duration (3 Weeks)	0.54	0.29		
Male size (standardised)	-0.25	0.13	4.06 (1)	0.04*
Female size (standardised)	0.66	0.18	13.68 (1)	0.0002***
Contest duration (1 Week) x Female size	-0.50	0.27	8.97 (2)	0.01*
Contest duration (3 Weeks) x Female size	-0.81	0.28		
2. Successful attempts				
Intercept (Loser, 1 Day)	-0.70	0.26		
Contest treatment (Winner)	0.54	0.22	6.00 (1)	0.01*
Contest duration (1 Week)	-0.04	0.28	12.13 (2)	0.002**
Contest duration (3 Weeks)	0.78	0.28		
Male size (standardised)	-0.39	0.14	7.88 (1)	0.005**
Female size (standardised)	0.10	0.13	0.56 (1)	0.46
3. Time with female				
Intercept (Loser, 1 Day)	4.17	0.17	600.22 (1)	<0.0001***
Contest treatment (Winner)	0.70	0.24	8.54 (1)	0.003**
Contest duration (1 Week)	0.27	0.24	1.41 (2)	0.49
Contest duration (3 Weeks)	0.07	0.25		
Male size (standardised)	-0.41	0.17	6.21 (1)	0.013*
Female size (standardised)	0.15	0.07	4.68 (1)	0.031*
Contest treatment (W) x Contest duration (1 Week)	-0.37	0.34	4.64 (2)	0.10
Contest treatment (W) x Contest duration (3 Weeks)	0.38	0.35		
Contest treatment (W) x Male size	0.43	0.23	3.52 (1)	0.061
Contest duration (1 Week) x Male size	0.61	0.26	6.44 (2)	0.04*
Contest duration (3 Weeks) x Male size	0.00	0.32		
Contest (W) x Duration (1 Week) x Male size	-0.97	0.34	8.06 (2)	0.018*
Contest (W) x Duration (3 Weeks) x Male size	-0.38	0.44		
4. Aggression towards rival				
Intercept (Loser, 1 Day)	0.77	0.27		
Contest treatment (Winner)	0.32	0.22	2.11 (1)	0.15
Contest duration (1 Week)	0.82	0.27	13.63 (2)	0.001**
Contest duration (3 Weeks)	0.91	0.28		
Male size (standardised)	-0.06	0.13	0.22 (1)	0.64
Female size (standardised)	0.09	0.13	0.49 (1)	0.48

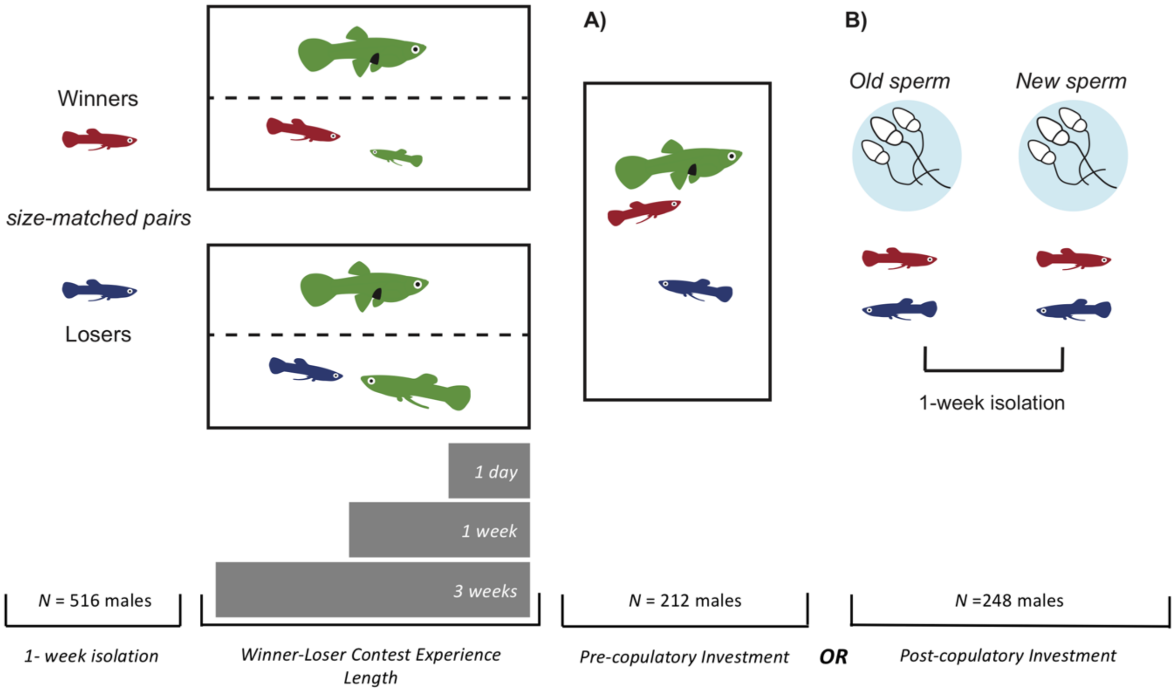

Figure 1. Experimental design. Male pairs were size-matched and randomly assigned to have a winning (red) or losing (blue) experience for either 1 day, 1 week or 3 weeks. Winners and losers were experimentally created by pairing focal males with smaller or larger rivals, respectively. A female was present behind a mesh barrier to motivate agonistic interactions between males. Experimental contests were staggered so that each experience treatment ended on the same day. At the end of the contest period, winners and losers from the same contest duration treatment were either (A) allowed to compete directly for a female for 20 mins to measure pre-copulatory investment, or (B) had their post-copulatory (sperm) traits measured. Post-copulatory investment males were stripped of their sperm immediately following contests to obtain baseline measures (old sperm), were isolated for seven days, and then had their replenished sperm traits measured (new sperm).

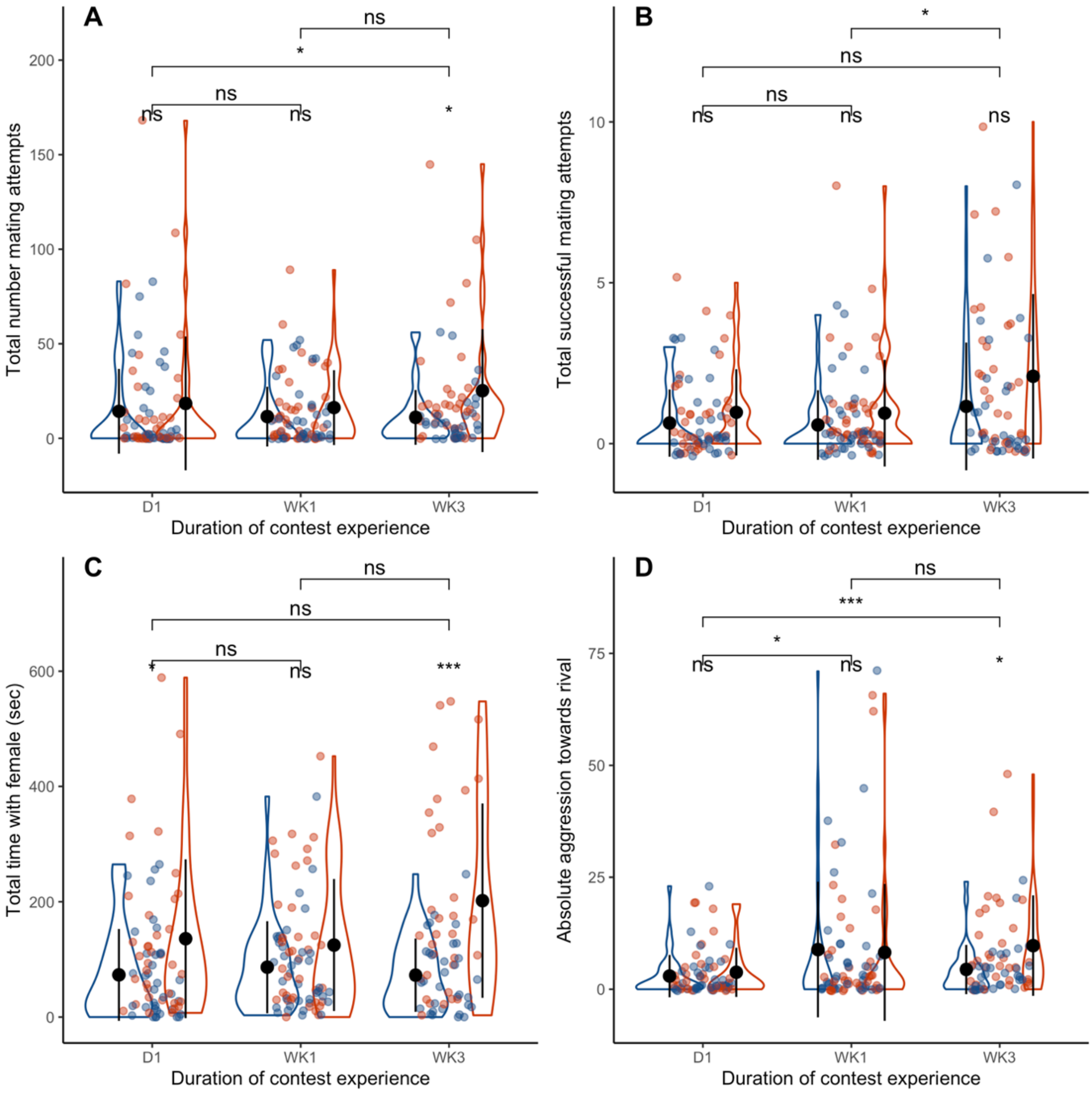

Figure 2. Pre-copulatory performance of winners (red) and losers (blue) following 1 day, 1 week or 3 weeks of contest experience. Winners made more mating attempts (A), were more successful at mating (B), and spent more time near the female (C). Winners and losers increased the number of aggressive interactions directed towards each other as the length of their contest experience increased (D). Violin plots show sample distribution with mean and standard deviation shown in black. Asterisks (*) above each contest duration treatment indicate significant differences between winners and losers, while lines with asterisks indicate significant differences across treatment durations (pairwise comparisons; ns = no significant difference). Statistical significance for pairwise comparisons were obtained using t-tests with the R package *ggpubr*.

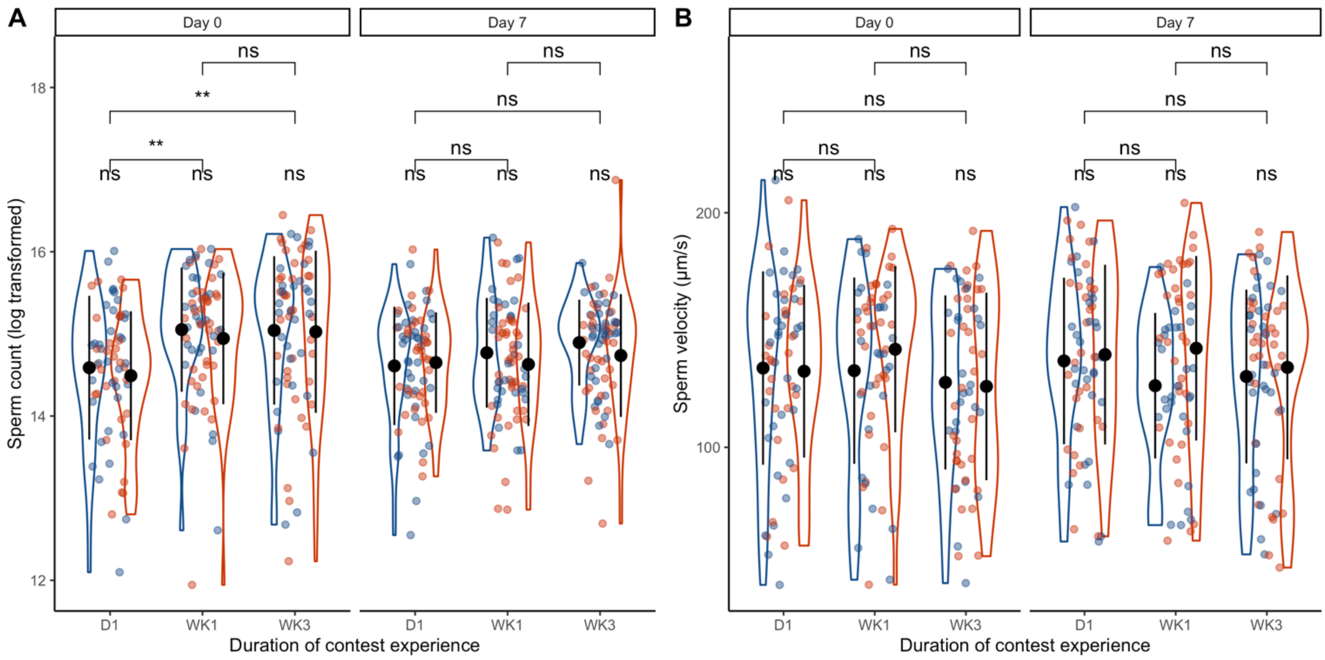

Figure 3. Violin plots depicting the old (Day 0) and replenished (Day 7) sperm traits of winners (red) and losers (blue) after 1 day, 1 week or 3 weeks of contest experience. Males had more sperm immediately after 1 or 3 weeks of contests than they did after 1 day of contests (A), but there were no differences between winners and losers in their old or replenished sperm counts. Males all had similar sperm velocity (VCL) immediately after their contest experiences (B), but winners had significantly faster replenished sperm velocity than losers (C). Asterisks (*) above each contest duration treatment indicate significant differences between winners and losers, while lines with asterisks indicate significant differences across treatment durations (pairwise comparisons; ns = no significant difference). Mean and standard deviation shown in black. Statistical significance for pairwise comparisons were obtained using t-tests with the R package *ggpubr*.

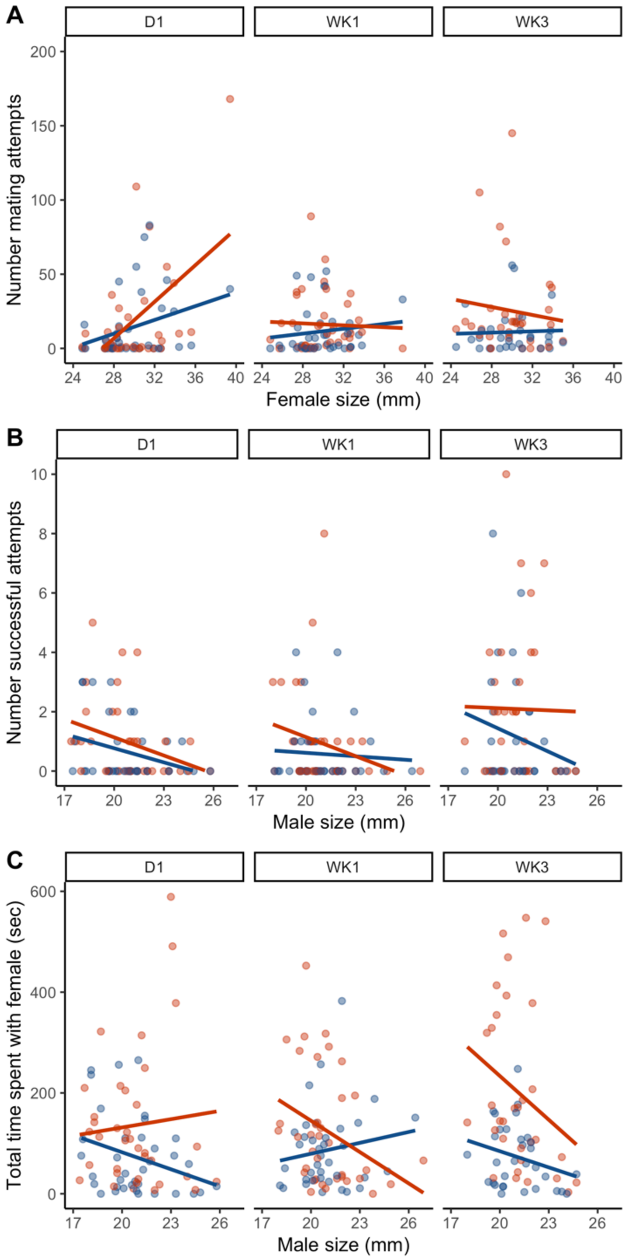

Figure 4. Simple linear regression lines show interactions between female body size (A), or male body size (B, C) and the pre-copulatory performance of winners (red) and losers (blue) after 1 day, 1 week or 3 weeks of contest experience. For the number of male mating attempts (A), larger females receive more harassment but only for males in the 1 day contest treatment. For the number of successful male mating attempts (B), larger males tended to be less successful than smaller males, and males in the 3 week treatment were more successful. Finally, the time each male spent with the female (C) was influenced by both his size, being a winner or loser, and the duration of his prior contest experience.