

# 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:** Fight outcomes often affect male fitness by determining their access to mates. Thus, “winner-loser” effects, where winners often win their next contest while losers tend to lose, can influence how males allocate resources toward pre- and postcopulatory traits. We experimentally manipulated the winning/losing experiences of pairs of size-matched male *Gambusia holbrooki* for 1 day, 1 week, or 3 weeks to test whether prior winning/losing experiences differentially affect the plasticity of male investment into either mating effort (precopulatory) or ejaculates (postcopulatory). When winner/loser pairs directly competed for a female, winners had better precopulatory outcomes than losers for three of the four traits we measured: mating attempts, successful attempts, and time spent with the female (but not aggression). However, winners and losers did not differ in either total sperm counts or sperm velocity. Interestingly, absolute male size, an important predictor of fighting success, mediated winner-loser effects on how long males then spent near a female. Compared with 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.

**Keywords:** winner-loser effect, sexual selection, sperm competition, behavior, plasticity, contests.

## Introduction

Male-male sexual competition is often a major determinant of male fitness. Competition among males for access to mates generates precopulatory sexual selection that

favors greater investment in traits associated with mating effort, such as 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 fertilize eggs (Parker 1970; Parker and Pizzari 2010). Postcopulatory sexual selection (e.g., sperm competition) favors male traits that increase ejaculate 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 for 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 into ejaculate competitiveness than would 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 postcopulatory 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; Dougherty et al. 2022). This

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trade-off is, however, likely to vary across environments or social settings. For example, when intense male-male competition prevents males from monopolizing females, higher levels of multiple mating by females shifts male allocation strategies from mating effort toward increased ejaculate 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 postcopulatory sexual selection can change. Such interactions between external factors and a male's state can favor phenotypic plasticity in male reproductive strategies for resource allocation (Bretman et al. 2011; Dore et al. 2018, 2020; Fox et al. 2019a). 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 postcopulatory 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 have shown 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 again (Hsu and Wolf 2001). Such “winner-loser” effects persist even when intrinsic differences in fighting ability or motivation, known as resource holding potential (RHP; Parker 1974; Arnott and Elwood 2008), are absent. That is, winners and losers have different carryover effects influencing their future fighting behavior 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 means that many fights are between closely matched individuals (see contest strategies reviewed in Chapin et al. 2019). 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 carryover 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 behaviors 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 postcopulatory 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 only a single contest, suggest that losers might switch to greater investment into traits under postcopulatory 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 into 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 postcopulatory 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 ejaculate 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 and hence the relative gains from further shifts in investment into traits under pre- and postcopulatory 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 ejaculate competitiveness. To date, few studies have experimentally manipulated the contest experience of males to determine how it affects investment into traits under pre- and postcopulatory sexual selection (but see Filice and Dukas 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)—affects 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 postcopulatory 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 1 day, 1 week, or 3 weeks, to test whether winning affects (a) how males allocate resources to traits under either pre- or postcopulatory sexual selection, (b) whether the duration of a male's contest experience leads to differences in relative allocation to traits under either pre- or postcopulatory sexual selection, 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* is a promiscuous poeciliid species that naturally forms high-density mixed-sex shoals. Male mosquitofish are aggressive toward each other, and larger males are socially dominant (Caldwell and Caldwell 1962; McPeck 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 (McPeck 1992). Winners spend more time associating with females than do losers (i.e., guarding her from other males; see Harrison et al. 2018), which suggests that winners, over time, 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 fertilize eggs (e.g., Zane et al. 1999). Males harass females and force copulations by swinging their gonopodium (modified anal fin) forward before thrusting it toward 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., Pilaastro et al. 1997; Booksmythe et al. 2013; Head et al. 2015).

### *Animal Collection and Maintenance*

Mature adult mosquitofish (identified by a hooklike tip to the gonopodia of males and by a gravid spot in females) were wild caught in Canberra, Australia (35°14'30.1"S, 149°06'17.0"E) during the summer of 2020–2021 (December–February). Fish were brought back to dedicated aquarium facilities at the Australian National University and housed in same-sex stock aquariums (90 L; ~50 individuals/aquarium) at 28°C ± 1°C under a 14L:10D photoperiod. Stock fish were fed ad lib. with commercial fish flakes, and experimental fish were given *Artemia salina* nauplii ad lib. 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 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 postcopulatory 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 precopulatory interactions for only 10 min. 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 anesthetized briefly in an ice slurry for 30 s to measure their standard body length with dial callipers and to tag them with a subcutaneous elastomer tag (Northwest Marine Technology, Anacortes, WA) for identification (a process that takes only 30 s). Focal male standard body length ranged from 17.4 to 26.9 mm (mean  $\pm$  SD:  $21.02 \pm 1.81$  mm). Males were then kept in individual 1-L tanks for 1 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 a larger (losers) competitor male (see Harrison et al. 2018; fig. 1). Size differences are an important determinant of social dominance in mosquitofish (Caldwell and Caldwell 1962). By randomly assigning matched focal males of the same size to become winners or losers, we could eliminate intrinsic differences in RHP (Parker 1974; Arnott and Elwood 2008) between winners and losers. Based on visual assessment, size differences between focal and competitor males were usually  $\sim 3$  mm but likely ranged between 1 and 7 mm.

Focal males either won or lost contests for 1 day, 1 week, or 3 weeks (fig. 1). These contest durations were chosen to reflect a range of winning-losing scenarios under which plasticity in allocation toward pre- and postcopulatory traits could occur. We expected focal males that competed against only a single rival in the 1-day treatment would show plasticity only in their mating behavior and not in their sperm traits. However, focal males that face new rivals over the course of the 3-week treatment would have enough time to alter sperm production and ejaculate traits. If males do plastically adjust their resource allocation on the basis of their prior contest experience, then we expected to see differences in male investment into mating effort versus ejaculate traits after 3 weeks. We then chose 1 week as a good midpoint between 1 day and 3 weeks to look at patterns of plasticity in response to prior contest experiences. Since male mosquitofish spend a large proportion of their time fighting each other and harassing females, it is reasonable to expect some males to experience consistent wins or losses for 3 weeks or far longer in the wild. Although the precise experience of only meeting approximately nine rivals over 3 weeks is unlikely in nature, the experience of constantly competing for 3 weeks is not. We therefore maximized the number of rivals a focal male faced within the constraint of logistics.

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). Con-

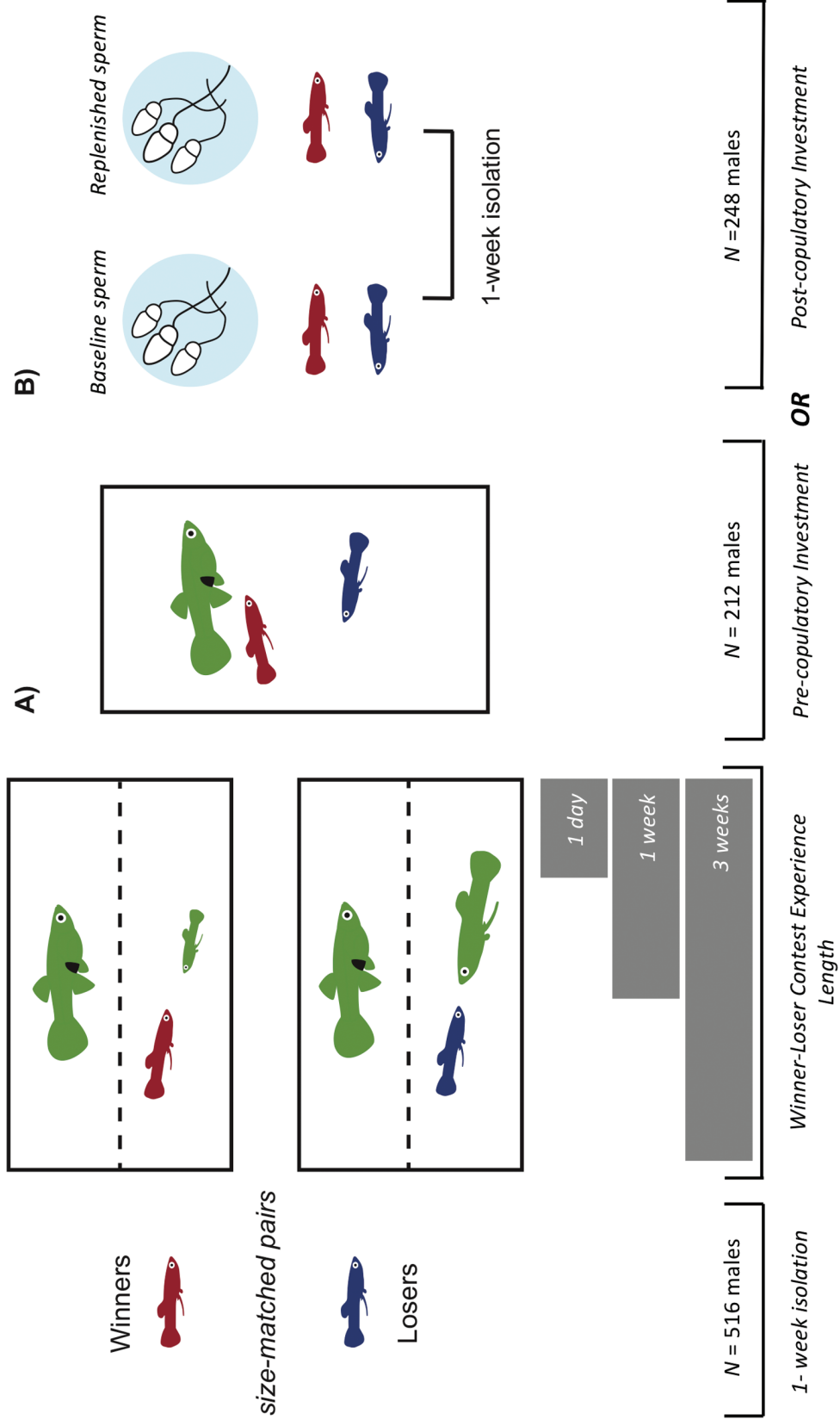
test experiences were broken up into 20 blocks to measure precopulatory investment and 21 blocks to measure postcopulatory investment. Each block had two sets of the three winner/loser duration treatments ( $N = 12$  pairs per block). In each winning/losing trial, a focal and a competitor male interacted 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 aquariums contained gravel and plastic plants, and they were lined with black plastic to minimize outside disturbance. Competitor males were rotated approximately every 3 days to ensure that focal males were continually winning/losing contests, while stimulus females were rotated every 7 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 either to compete directly for a female to measure precopulatory investment or to have their sperm traits measured (postcopulatory investment; fig. 1).

#### Mortality

Contest treatments ended with fewer than the intended 40 males for each of the six winner/loser-by-contest duration combinations because of natural mortality. Twelve of the 516 males ( $\sim 2\%$  of our total sample size) died during the first isolation period posttagging 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 ( $\chi^2 = 4.33$ ,  $df = 1$ ,  $P = .037$ ), and mortality also differed between the three contest duration treatments ( $\chi^2 = 9.69$ ,  $df = 2$ ,  $P = .008$ ). It was highest for the 3-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 toward losers that could better survive in a challenging competitive environment.

#### Precopulatory Investment

To compare male investment into precopulatory mating behaviors, we placed size-matched (paired  $t$ -test: mean difference = 0.01 mm,  $t = 0.54$ ,  $df = 105$ ,  $P = .594$ ) 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 used only once. Male interactions were observed for 20 min where we recorded (a) time spent near the female, (b) number of mating attempts, (c) number of successful mating attempts, and (d) aggression directed toward the rival. Mating attempts



**Figure 1:** Experimental design. Male pairs were size matched and randomly assigned to have a winning (red) or losing (blue) experience for 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 allowed to compete directly for a female for 20 min to measure precopulatory investment (A) or had their postcopulatory (sperm) traits measured (B). Postcopulatory investment males were stripped of their sperm immediately following contests to obtain baseline measures, were isolated for 7 days, and then had their replenished sperm traits measured.

were recorded each time a male swung his gonopodium forward toward 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 behaviors of 36 pairs of 1-day-treatment males, 38 pairs of 1-week-treatment males, and 32 pairs of 3-week-treatment males.

#### Postcopulatory Investment

To compare male investment into postcopulatory 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 7 days later to measure the effect of winning/losing on rates of sperm replenishment or sperm traits. Sperm collected immediately after 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).

**Sperm Collection.** At the end of their contest experiences, focal males were anesthetized 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 7-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 100–1,100  $\mu\text{L}$  of extender medium (pH 7.5 with composition: 207 mM NaCl, 5.4 mM KCl, 1.3 mM  $\text{CaCl}_2$ , 0.49 mM  $\text{MgCl}_2$ , 0.41 mM  $\text{MgSO}_4$ , 10 mM Tris [Cl]) to count sperm. Sperm collection and subsequent trait measurements were performed masked to male contest treatment.

**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  $\mu\text{L}$  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) under 100 $\times$  magnification. Threshold values defining cell detection were predetermined as elongation percentage 15–65 and head size 5–15  $\mu\text{m}$  (without the static tail filter; 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 rptR (Stoffel et al. 2017). Sperm subsample counts for each male were highly repeatable on both day 0 ( $R = 0.90$ ; 95% confidence interval: 0.88–0.93;  $P < .001$ ) and day 7 ( $R = 0.84$ ; 95% confidence interval: 0.81–0.87;  $P < .001$ ). 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 baseline and replenished sperm, respectively.

**Sperm Velocity.** To measure sperm velocity, we used two samples from each male's ejaculate (three sperm bundles each in 3  $\mu\text{L}$  of extender medium). We then pipetted each sample onto the center of a cell of a 12-cell multitest slide (MP Biomedicals, Aurora, OH) previously coated with 1% polyvinyl alcohol solution to prevent sperm from sticking to the slide. Each sample was then "activated" with 3  $\mu\text{L}$  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  $\mu\text{m}/\text{s}$  for VAP and 15  $\mu\text{m}/\text{s}$  for VCL (Gasparini et al. 2010, 2013; Chung et al. 2021). Our measures of VAP and VCL were highly correlated (Pearson's  $r = 0.99$ ,  $N = 372$ ,  $P < .001$ ). 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 baseline sperm and 190 males for replenished sperm.

#### Statistical Analyses

**Precopulatory Investment.** We fitted generalized 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 precopulatory 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 toward 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.

*Postcopulatory Investment.* For postcopulatory investment, we fitted 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 measurement (baseline vs. replenished sperm). These models had sperm count (log transformed) and sperm velocity (VCL) as the response variable and contest experience, contest duration, sperm measurement, and male body size, as well as 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., baseline and replenished sperm). We present the reduced models with main effects and significant interaction terms in table 2.

*Precopulatory 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 precopulatory mating behavior (e.g., Harrison et al. 2018), but our main focus was on maximizing 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 fitted GLMMs (negative binomial error with log-link function) for each of the four precopulatory traits. These models had contest experience, contest duration, and their interaction as fixed categorical factors and also included male and female body size (both centered and standardized 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,

**Table 1:** Effects of prior winning or losing experiences (and the duration of these experiences) on male precopulatory mating effort

	Estimate $\pm$ SE	$\chi^2$ (df)
Mating attempts:		
Intercept (loser, 1 day)	2.09 $\pm$ .32	
Contest treatment (winner)	.47 $\pm$ .21	4.90 (1)*
Contest duration (1 week)	.13 $\pm$ .32	1.76 (2)
Contest duration (3 weeks)	.43 $\pm$ .34	
Successful attempts:		
Intercept (loser, 1 day)	-.57 $\pm$ .26	
Contest treatment (winner)	.52 $\pm$ .22	5.63 (1)*
Contest duration (1 week)	-.13 $\pm$ .28	9.45 (2)**
Contest duration (3 weeks)	.63 $\pm$ .27	
Time spent with female:		
Intercept (loser, 1 day)	4.28 $\pm$ .14	
Contest treatment (winner)	.65 $\pm$ .15	19.62 (1)***
Contest duration (1 week)	.05 $\pm$ .18	1.43 (2)
Contest duration (3 weeks)	.21 $\pm$ .18	
Aggression toward rival:		
Intercept (loser, 1 day)	.83 $\pm$ .25	
Contest treatment (winner)	.30 $\pm$ .20	2.25 (1)
Contest duration (1 week)	.74 $\pm$ .29	9.92 (2)**
Contest duration (3 weeks)	.85 $\pm$ .30	

Note: Estimates and standard errors are from reduced generalized linear mixed models. Summary statistics and significance are from type II ANOVAs. Models use negative binomial error distributions.

\*  $P < .05$ .

\*\*  $P < .01$ .

\*\*\*  $P < .001$ .

**Table 2:** Effects of prior winning or losing experiences (and the duration of these experiences) on male postcopulatory ejaculate traits

	Estimate $\pm$ SE	$\chi^2$ (df)
Total sperm count:		
Intercept (loser, 1 day, baseline sperm)	10.51 $\pm$ 1.59	43.86 (1)***
Contest treatment (winner)	-.07 $\pm$ .08	.80 (1)
Contest duration (1 week)	.46 $\pm$ .13	18.91 (2)***
Contest duration (3 weeks)	.51 $\pm$ .13	
Sperm measure (replenished sperm)	.10 $\pm$ .11	.76 (1)
Male body size (standardized)	1.34 $\pm$ .52	6.60 (1)*
Contest duration (1 week) $\times$ sperm measure (replenished)	-.41 $\pm$ .15	7.87 (2)*
Contest duration (3 weeks) $\times$ sperm measure (replenished)	-.32 $\pm$ .15	
Sperm velocity (VCL):		
Intercept (loser, 1 day, baseline sperm)	109.30 $\pm$ 67.22	
Contest treatment (winner)	5.12 $\pm$ 3.15	2.62 (1)
Contest duration (1 week)	1.67 $\pm$ 3.88	2.60 (2)
Contest duration (3 weeks)	-4.34 $\pm$ 3.86	
Sperm measure (replenished sperm)	6.38 $\pm$ 3.13	4.15 (1)*
Male body size (standardized)	3.96 $\pm$ 21.95	.01 (1)

Note: The total sperm count model includes significant interaction terms. Estimates and standard errors for the sperm velocity model are from the reduced model where nonsignificant interaction terms were removed. Summary statistics and significance are from a type III ANOVA for the sperm count model and a type II ANOVA for the sperm velocity model. Both models include male body size (standardized and centered to the mean) and baseline trait measures as fixed factors to test their effects on replenished ejaculate traits. Models use Gaussian error distributions.

\*  $P < .05$ .

\*\*\*  $P < .001$ .

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 criterion tables to identify the best-fitting model (for model fitting, see code provided in Harrison et al. 2022). We removed all nonsignificant 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 whether 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  $\chi^2$  tests in the reduced models and type III in the models that include interaction terms. We set  $\alpha = .05$  for all model terms except three-way interaction terms (where  $\alpha$  was .01). All tests were two-tailed. Analyses reported in this article can be reproduced using the data and code provided in Harrison et al. (2022).

## Results

### *Precopulatory Investment*

Surprisingly, we found no significant interactions between contest experience and prior contest duration for any of the four precopulatory traits we measured, suggesting that plasticity in allocation toward precopulatory mating be-

haviors does not change with the duration of a male's contest experience (table 1). Winners and losers differed for three of the four traits we measured (fig. 2). When winners and losers directly competed for a female, winners made significantly more mating attempts ( $\chi^2 = 4.90$ ,  $df = 1$ ,  $P = .027$ ; fig. 2A), more often made successful mating attempts ( $\chi^2 = 5.63$ ,  $df = 1$ ,  $P = .018$ ; fig. 2B), and spent more time near the female ( $\chi^2 = 19.62$ ,  $df = 1$ ,  $P < .0001$ ; fig. 2C) than losers. However, winners and losers did not differ significantly in how many aggressive interactions they initiated ( $\chi^2 = 2.25$ ,  $df = 1$ ,  $P = .134$ ; fig. 2D).

Prior contest experience duration had no significant effect on the number of mating attempts ( $\chi^2 = 1.76$ ,  $df = 2$ ,  $P = .414$ ) or on the time spent near the female ( $\chi^2 = 1.43$ ,  $df = 2$ ,  $P = .488$ ). However, the number of successful attempts made ( $\chi^2 = 9.45$ ,  $df = 2$ ,  $P = .009$ ) and male aggression toward each other ( $\chi^2 = 9.92$ ,  $df = 2$ ,  $P = .007$ ) 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).

### *Postcopulatory Investment*

When considering both baseline and replenished sperm measures, there were no significant interactions between



**Table 3:** Effects of interactions between male and female body size, contest experiences, and contest durations on male precopulatory mating effort

	Estimate $\pm$ SE	$\chi^2$ (df)
Mating attempts:		
Intercept (loser, 1 day)	2.11 $\pm$ .24	74.93 (1)***
Contest treatment (winner)	.40 $\pm$ .21	3.77 (1)*
Contest duration (1 week)	.20 $\pm$ .25	3.60 (2)
Contest duration (3 weeks)	.54 $\pm$ .29	
Male size (standardized)	-.25 $\pm$ .13	4.06 (1)*
Female size (standardized)	.66 $\pm$ .18	13.68 (1)***
Contest duration (1 week) $\times$ female size	-.50 $\pm$ .27	8.97 (2)*
Contest duration (3 weeks) $\times$ female size	-.81 $\pm$ .28	
Successful attempts:		
Intercept (loser, 1 day)	-.70 $\pm$ .26	
Contest treatment (winner)	.54 $\pm$ .22	6.00 (1)*
Contest duration (1 week)	-.04 $\pm$ .28	12.13 (2)**
Contest duration (3 weeks)	.78 $\pm$ .28	
Male size (standardized)	-.39 $\pm$ .14	7.88 (1)**
Female size (standardized)	.10 $\pm$ .13	.56 (1)
Time spent with the female:		
Intercept (loser, 1 day)	4.17 $\pm$ .17	600.22 (1)***
Contest treatment (winner)	.70 $\pm$ .24	8.54 (1)**
Contest duration (1 week)	.27 $\pm$ .24	1.41 (2)
Contest duration (3 weeks)	.07 $\pm$ .25	
Male size (standardized)	-.41 $\pm$ .17	6.21 (1)*
Female size (standardized)	.15 $\pm$ .07	4.68 (1)*
Contest treatment (W) $\times$ contest duration (1 week)	-.37 $\pm$ .34	4.64 (2)
Contest treatment (W) $\times$ contest duration (3 weeks)	.38 $\pm$ .35	
Contest treatment (W) $\times$ male size	.43 $\pm$ .23	3.52 (1)
Contest duration (1 week) $\times$ male size	.61 $\pm$ .26	6.44 (2)*
Contest duration (3 weeks) $\times$ male size	.00 $\pm$ .32	
Contest (W) $\times$ duration (1 week) $\times$ male size	-.97 $\pm$ .34	8.06 (2)*
Contest (W) $\times$ duration (3 weeks) $\times$ male size	-.38 $\pm$ .44	
Aggression toward rival:		
Intercept (loser, 1 day)	.77 $\pm$ .27	
Contest treatment (winner)	.32 $\pm$ .22	2.11 (1)
Contest duration (1 week)	.82 $\pm$ .27	13.63 (2)**
Contest duration (3 weeks)	.91 $\pm$ .28	
Male size (standardized)	-.06 $\pm$ .13	.22 (1)
Female size (standardized)	.09 $\pm$ .13	.49 (1)

Note: Mating attempts and time spent with female models use type III ANOVAs for summary statistics, while models for successful attempts and aggression toward rival use type II ANOVAs. All models include female and male body size (standardized and centered to the mean) as fixed factors. Where interactions were nonsignificant, they were dropped from the model. Models use negative binomial error distributions.

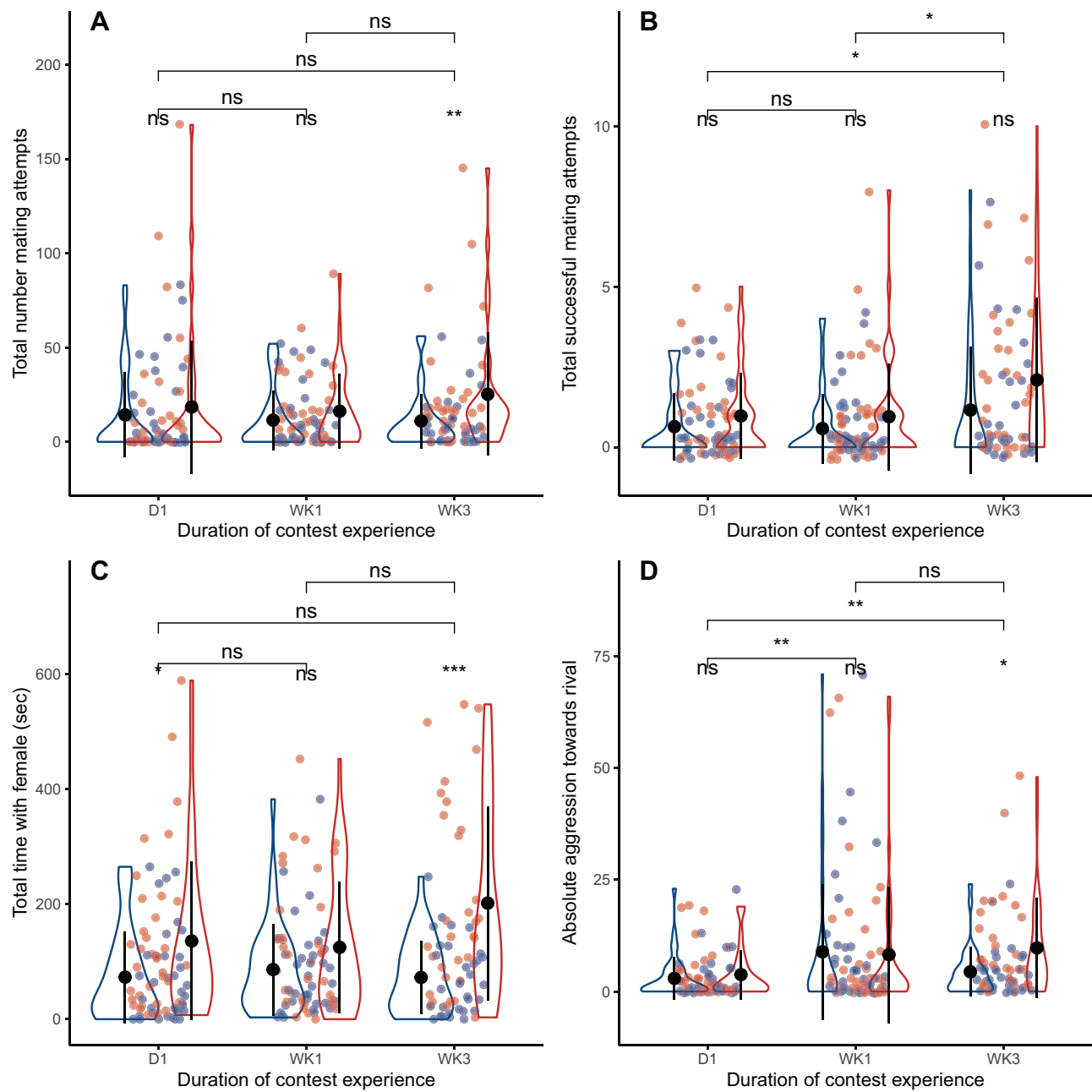
\*  $P < .05$ .

\*\*  $P < .01$ .

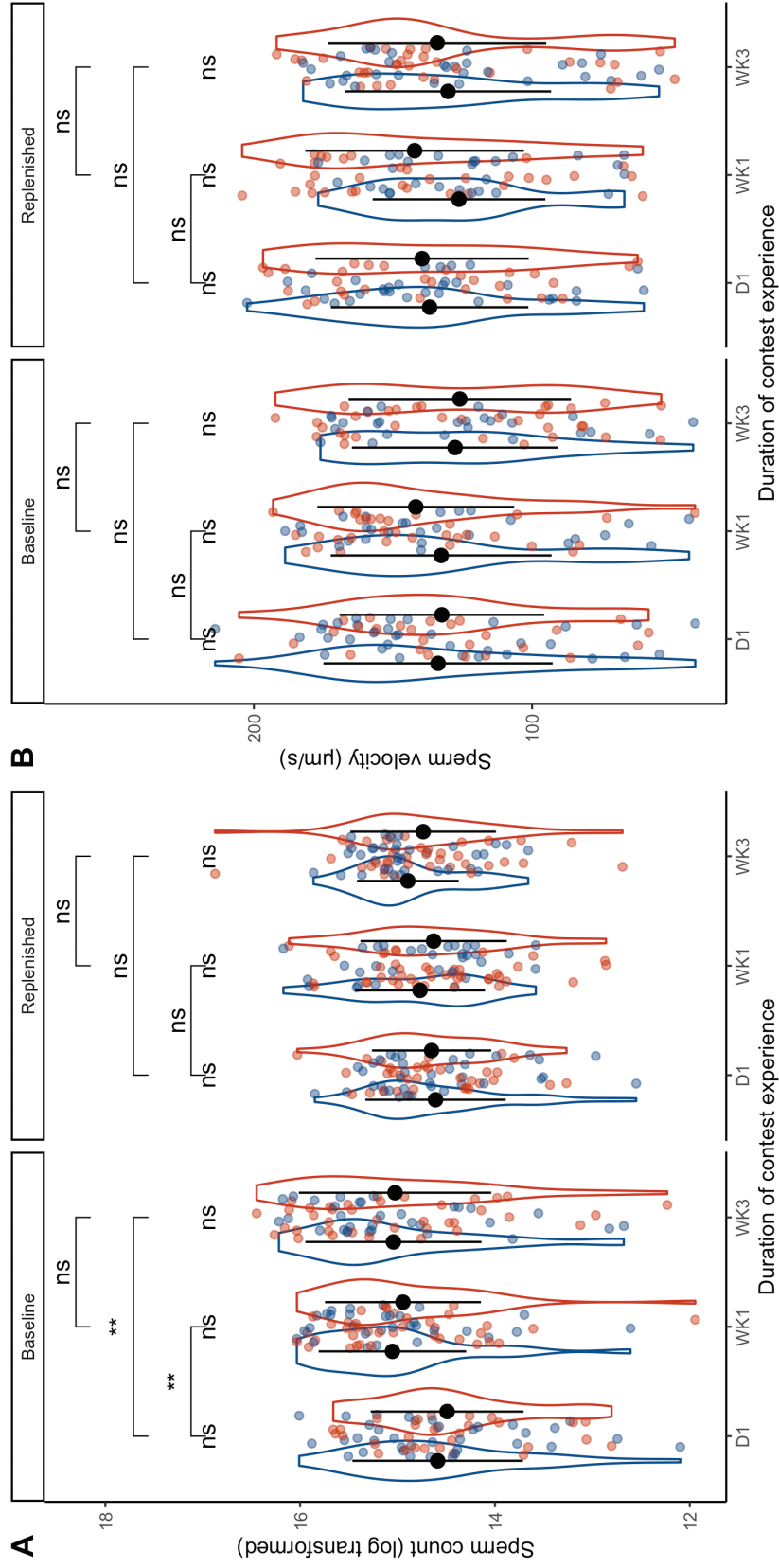
\*\*\*  $P < .001$ .

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:  $\chi^2 = 0.80$ ,  $df = 1$ ,  $P = .371$ ; fig. 3A) or sperm velocity ( $\chi^2 = 2.62$ ,  $df = 1$ ,  $P = .106$ ; fig. 3B). For sperm counts, there was, however, a significant interaction between sperm measure (baseline vs. replenished

sperm) and the duration of the prior contest experience ( $\chi^2 = 7.87$ ,  $df = 2$ ,  $P = .020$ ). Males that experienced only 1 day of contests replenished their sperm stores to baseline levels, while males that experienced either 1 or 3 weeks of contests did not (table 2). Replenished sperm was significantly faster than baseline sperm ( $\chi^2 = 4.15$ ,  $df = 1$ ,  $P = .042$ ), but there was no effect of contest



**Figure 2:** Precopulatory 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 toward each other as the length of their contest experience increased (D). Violin plots show sample distributions with means and standard deviations 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; \* $P < .05$ ; \*\* $P < .01$ ; \*\*\* $P < .001$ ; 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 baseline and replenished 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 baseline or replenished sperm counts. Males all had similar sperm velocity (VCL) both immediately after their contest experiences and when their replenished sperm was measured (B). 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;  $**P < .01$ ; ns = no significant difference). Means and standard deviations are shown in black. Statistical significance for pairwise comparisons were obtained using *t*-tests with the R package ggpubr.

treatment duration on sperm velocity ( $\chi^2 = 2.60$ ,  $df = 2$ ,  $P = .272$ ; table 2).

#### *Precopulatory Traits and Their Interactions with Body Size*

Compared with males that had only 1 day of contest experience, males that experienced 1 or 3 weeks of contests made significantly fewer mating attempts toward larger females ( $\chi^2 = 8.97$ ,  $df = 2$ ,  $P = .011$ ; fig. 4A). In addition, smaller males more often made successful mating attempts than did larger males ( $\chi^2 = 7.88$ ,  $df = 1$ ,  $P = .005$ ; fig. 4B).

Interestingly, there was a significant three-way interaction among contest treatment, contest duration, and male body size that affected how long a male spent near the female ( $\chi^2 = 8.80$ ,  $df = 2$ ,  $P = .012$ ; table 3). How a male responded to his contest experience and its duration was moderated by his body size (fig. 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:  $\chi^2 = 0.22$ ,  $df = 1$ ,  $P = .639$ ; female size:  $\chi^2 = 0.49$ ,  $df = 1$ ,  $P = .482$ ; table 3).

#### **Discussion**

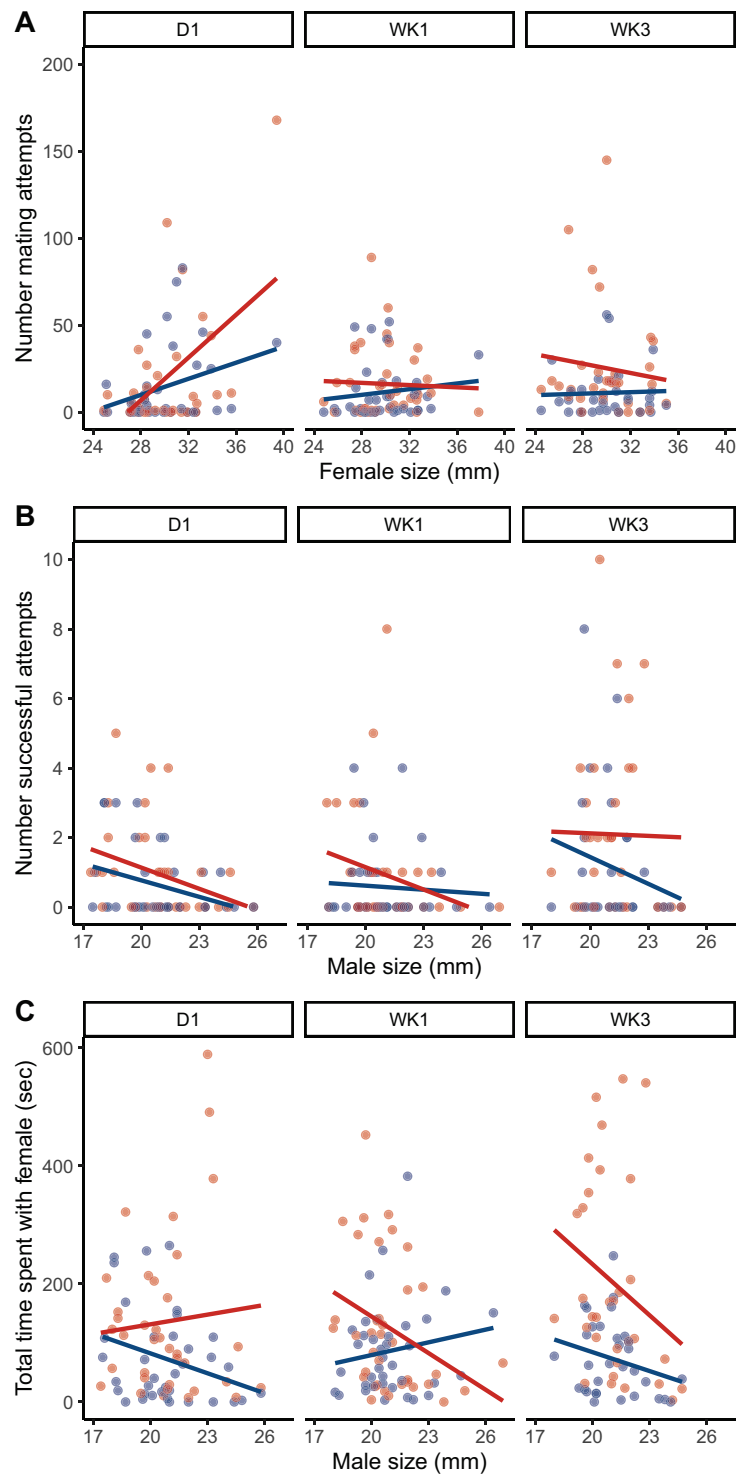
Since the outcome of male-male contests can determine access to both females and resources, it is plausible that winners and losers plastically adjust their investment into condition-dependent sexually selected traits. To test this, we manipulated the extent to which male mosquitofish (*Gambusia holbrooki*) 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 postcopulatory sexual selection. Winners did indeed have greater precopulatory success than losers for three of the four behavioral 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, losers did not invest relatively more into traits under postcopulatory sexual selection: there was no effect of winning or losing on either sperm count or sperm velocity. This finding is surprising, as males with poor fighting abilities are widely predicted to produce larger amounts and/or faster 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 postcopulatory sexual selection to favor 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 ARTs (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 whether 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 1 day, 1 week, or 3 weeks before we measured putative sexually selected traits (Bisazza and Marin 1995; O'Dea et al. 2014; Fox et al. 2019b). Although there was a difference between winners and losers in behaviors that affect mating success, losers did not appear to reallocate resources to traits under postcopulatory 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 males do not 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 toward different aspects of reproduction. *Gambusia holbrooki* males survive for only one breeding season (Kahn et al. 2013). As such, plastic adjustments in reproductive allocation by



**Figure 4:** Simple linear regression lines showing interactions between female body size (A) or male body size (B, C) and the precopulatory 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.

males in *G. holbrooki* and other short-lived species can affect fitness only in the current breeding season (e.g., semelparous marsupials; Fisher et al. 2013). Winning and losing experiences might therefore favor shifts in other life history traits, such as growth rates or longevity, that have larger effects on fitness than small changes in allocation between ejaculate traits 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 with yellow-headed males that invest into sperm competition (Rollings et al. 2017). Similar trade-offs between reproductive effort and life span occur in other species with continuous variation in reproductive tactics (e.g., Lemaître et al. 2015, 2020).

Second, male coercive mating is an “activational” behavior (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 behavioral traits, such as mating (Lane and Briffa 2022). There is weaker evidence that winning or losing fights affects ejaculate traits (e.g., Filice and Dukas 2019; Tunj 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 directly 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 improve sperm performance.

Third, if the energetic costs of mating competition or sperm production are low, there might be minimal trade-offs between traits under pre- and postcopulatory sexual selection (i.e., mating effort and ejaculates; Parker et al. 2013). However, if male-male competition is such that males cannot readily monopolize females, all males tend to invest more into ejaculate traits that increase their fertilization success under sperm competition because of

higher marginal fitness gains (Lüpold et al. 2014). We found that male *G. holbrooki* adjusted only precopulatory traits following a winning experience. It is worth noting, however, that trade-offs are obscured when looking across individuals if there is high interindividual variation in resource acquisition (van Noordwijk and De Jong 1986). That is, we may have been unable to observe differences between winners and losers if both contest experiences favored greater investment into postcopulatory traits. 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 with mating behavior (Chung et al. 2021). It is possible, then, that winners allocate the additional energetic resources gained from greater access to food toward both pre- and postcopulatory 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 future social environment (Dore et al. 2018). For instance, if males do not keep count of prior wins or losses or if 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. 1998). 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 favor always investing into ejaculate competitiveness (e.g., the size-based mating tactics of sailfin mollies [*Poecilia latipinna*]; 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 toward 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 precopulatory 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 these smaller males. 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 2022). 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 toward 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 visualization, and interpretation. R.V.-T. and M.D.J. contributed to data analysis and 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 approved the final version submitted for publication.

### Data and Code Availability

All data and R code associated with this study have been made available via the Dryad Digital Repository (<https://doi.org/10.5061/dryad.fttdz08vn>; Harrison et al. 2022).

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Symposium Editor: Suzanne H. Alonzo



“The nature of lava overflows and the causes of the differences in their rate of motion are well discussed. The accompanying illustration . . . of a lava stream which, from its imperfect fluidity in flowing over the edge of a precipice, forms heavy pendant masses like a ‘guttering’ candle, is a fair example of the quality of the woodcuts.” Figured: “Cascade of lava tumbling over a cliff in the Island of Bourbon.” From the review of “Volcanoes” (*The American Naturalist*, 1882, 16:492–496).