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Winners have higher pre-copulatory mating success but losers have better post-copulatory outcomes

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In many animals, the outcomes of competitive interactions can have lasting effects that influence an individual's reproductive success and have important consequences for the strength and direction of evolution via sexual selection. In the fruit fly, *Drosophila melanogaster*, males that have won previous contests are more likely to win in subsequent conflicts and losers are more likely to lose (winner–loser effects), but the direct fitness consequences and genetic underpinnings of this plasticity are poorly understood. Here, we tested how male genotype and the outcomes of previous male–male conflicts influence male pre- and post-copulatory success. We quantified pre-copulatory success in a choice and no-choice context, and post-copulatory success by quantifying ejaculate offensive and defensive ability. We found that winners have higher reproductive success compared to losers in both pre-copulatory scenarios. However, losers consistently mated for a longer duration, boosted female fecundity and had an increased paternity share when they were the first males to mate, suggesting increased investment into post-copulatory mechanisms. Finally, by using clonal hybrids from the *Drosophila* Genetic Reference Panel, we documented that genetic variation explained a sizeable proportion of the observed differences between lines, and of the interaction between line and winner and loser effects. Our results place the behavioural data on winner–loser effects in an evolutionary context by documenting the potential fitness gain to males from altering their reproductive strategy based on fighting experience. Our data may also explain the presence and maintenance of trade-offs between different male reproductive strategies.

1. Introduction

Prior competitive interactions can have lasting effects across many species, where winners of previous contests are more likely to win in subsequent conflicts, and losers are more likely to lose [1,2]. Winning and losing also influence a variety of behaviours including those related to aggression, mating and exploration [3–5], and may thus have major consequences for fitness. However, our understanding of the evolutionary biology of these behavioural effects is limited [2]. Specifically, few studies have addressed the fitness consequences and genetic underpinnings of the behavioural plasticity associated with winner–loser effects.

When thinking about winner effects, the potential fitness benefits are rather intuitive, and studies indeed have found that winners typically enjoy increased access to resources and mates [6]. These benefits may be manifested through an improved ability to deter other males from access to mates (intrasexual selection), or through increased attractiveness to potential mates (intersexual selection). For example, in the mosquitofish, *Gambusia holbrooki*, winners spent significantly more time associating with females compared to losers when physical interactions between males were allowed. Since female cooperation is not necessary for mating in mosquitofish, this result suggests that the effect was largely due to the winner preventing losers from interacting

with females, or losers actively avoiding winners to reduce the potential costs of further fighting. The winners, however, did not make more copulation attempts or mate more often than the losers [7]. In other species, similar protocols have demonstrated that female choice may also influence the mating success of winners and losers. In field crickets, *Gryllus assimilis*, females were more likely to mount males who won a previous fight compared to males that lost a previous fight when presented to a single male, suggesting that the winners were more attractive [8]. Note, however, that, unlike the mosquitofish experiment, which randomly assigned males into winner and loser roles, the cricket study relied on natural male fighting outcomes, which implies selection bias [9]. Hence, in the cricket study, one cannot separate the effects of inherent male quality from the isolated effects of winning or losing.

Unlike winner effects, the evolutionary perspective of loser effects is not as clear. Some researchers suggest that individuals may benefit from the behavioural plasticity associated with losing by reducing the potential energetic costs and physical harm associated with future conflicts, but there is no clear evidence of how these modified behaviours could be maintained against the fitness costs of decreased access to resources and mating opportunities [2]. One solution to this paradox may involve the use of alternative mating tactics to maximize reproductive success. Specifically, losers may gain from strategically investing more resources into ejaculate traits if mating opportunities are few [10,11]. In the broad-horned flour beetle, *Gnathocerus cornutus*, males that lost a fight 24 h earlier transferred significantly more sperm during copulation compared with winners [12]. In another study using the same species, males that lost a fight switched to a dispersal strategy by leaving the fighting site, compared to winners who tended to remain on the same site after winning a fight [13]. Although the flour beetle studies involved selection bias, they suggest that losers choose their mating strategies to make the best of a bad situation. To the best of our knowledge, however, no one has tested the fitness outcomes associated with the behavioural changes that accompany winning and losing.

In fruit flies, *Drosophila melanogaster*, males engage in a variety of pre- and post-copulatory contests to acquire mates and maximize paternity. Male pre-copulatory success is influenced by a combination of acoustic, visual, chemical, and tactile signals [14] and courtship interference [15], and their post-copulatory success may be influenced by the amount of sperm and accessory gland proteins (Acps) that are transferred in the seminal fluid [16,17]. Given that males can employ a variety of pre- and post-mating strategies, each involving different benefits and costs, one would expect them to choose the strategy that would maximize fitness in their current social setting [10,11]. For example, male fruit flies housed in a vial with a conspecific rival subsequently mate for longer and sire significantly more offspring (whether they are the first or second males to mate) compared to males housed alone [18]. The authors suggest that males housed with rivals perceive a greater risk for sperm competition in their social environment, and thus invest more into tactics that will improve their post-copulatory success. Given that males adaptively alter their mating strategies based on their sociosexual environment, we expect winner–loser effects to have a significant effect

on the performance of males in pre- and post-copulatory competitions.

Here, we hypothesized that the reproductive success of winners and losers would differ, with winners having higher pre-copulatory success and losers achieving higher post-copulatory success. We used fruit flies to examine the effect of winning and losing on male reproductive success in (1) a choice pre-copulatory context, (2) a no-choice pre-copulatory context, (3) a defensive post-copulatory context and (4) an offensive post-copulatory context. We predicted that winners would have higher reproductive success in the pre-copulatory contexts (tests 1 and 2), while losers would have higher reproductive success in the post-copulatory competitions (tests 3 and 4). In addition, we used clonal hybrid lines to quantify the degree of genetic variation associated with these plastic effects. Understanding the degree to which individual genotype influences the magnitude of behavioural plasticity (and its fitness consequences) between winners and losers is of great interest to biologists because variation in male competitive and/or mating behaviours can influence the strength and direction of evolution via sexual selection [19].

2. Material and methods

(a) Fly stocks and general

We used 28 randomly selected lines from the *Drosophila* Genetic Reference Panel (DGRP). These lines were derived from wild flies caught in Raleigh, North Carolina, USA, and repeatedly inbred for 20 generations [20]. To alleviate the deleterious phenotypic effects associated with inbreeding, we generated hybrids by crossing each line to a standardized reference line, thereby creating unique hybrid clones (hereafter referred to as hybrids). Within hybrids, individuals are genetically identical, but between hybrids, individuals share an identical clonal haplotype inherited from their mother, and a unique clonal haplotype inherited from their father, allowing us to quantify the degree of genetic variation associated with phenotypic differences expressed from this unique haplotype.

To generate standardized competitors, we used descendants from the 'bully' population obtained from the Kravitz Lab (Harvard University, Cambridge, MA, USA). These flies have been artificially selected for increased male–male aggression by choosing the winners of fights over 34–37 generations [21]. To manipulate the amount of aggression expressed by these males towards focal males, we used either 1-day-old or 5-day-old males (hereafter referred to as young and mature bullies, respectively), because young males show little aggression compared to their older counterparts [22] (see below). One day before each test, we dusted all bully males with pink fluorescent powder to distinguish them from the focal males. We lightly tapped individual flies into vials containing sparse amounts of the powder, giving them ample time to recover for the experience phase on the following day.

To determine paternity success, we used flies derived from the *Ives* population (hereafter IV) obtained from the Long Lab (Wilfrid Laurier University, Waterloo, ON, Canada). Since its collection in 1975, this population has been maintained at large census size (greater than 1000 adults per generation), on non-overlapping generations on a standardized culture protocol [23]. A sub-population of these flies carries the recessive autosomal *bw* mutation [24] (hereafter referred to as IV-*bw*), resulting in a visible brown-eye colour phenotype in comparison to the standard red-eye wild-type individuals. As the expression of this phenotype is controlled by a single recessive allele, two

individuals who possess this phenotype will always produce offspring that express it, but any offspring from wild-type and brown-eye cross will appear wild-type.

We reared all experimental flies at a standardized density of about 100 eggs per vial containing approximately 5 ml of standard fly medium made of water, sucrose, cornmeal, yeast, agar and methyl paraben, and stored all flies in an incubator at 25°C and 60% relative humidity with a 12:12 h light:dark cycle. We collected newly eclosed flies within 8 h of eclosion under light CO₂ anaesthesia and housed males in individual vials and females in groups of 20 with a pinch of live yeast. Following their initial collection, we handled all flies using gentle aspiration. We conducted all trials in ‘aggression arenas’ consisting of Petri dishes 35 mm in diameter and 8 mm high. We covered the base of each arena with a circular piece of filter paper and placed at its centre an attractive food patch 7 mm in diameter and 3 mm high consisting of standard fly medium sprinkled with live yeast [22].

(b) Aggression tests

A major challenge in studying winner–loser effects is developing unbiased protocols that reliably generate focal winners and losers. Although this issue has been brought to attention in two major reviews [1,2], many studies continue to use self-selection protocols, which confound the effects of experience with intrinsic individual factors. To avoid self-selection biases, we randomly chose focal males of similar sizes and assigned them to either the winner or loser treatment. Focal males of the winner treatment interacted with young bullies, while focal males of the loser treatment interacted with mature bullies. As males are highly motivated to monopolize the attractive food patch in each arena by displaying aggression towards rival males [22], males that are more aggressive are consistently winners in this context [25]. In a preliminary experiment, we quantified the success of our treatments by recording aggression in matches between focal males and young bullies, and focal males and mature bullies. The focal males were derived from a recently established wild-caught population we collected in Hamilton, ON in 2015. In each trial, we placed a single 4-day-old male randomly selected from our base population into an aggression arena with either a 1-day-old bully (young) or a 5-day-old bully (mature) and video recorded the interactions for 30 min using an iPod Touch. Then an observer blind to bully age used the BORIS software [26] to record from the videos the total number and duration of aggressive behaviours displayed by both flies. Aggressive behaviours were defined by the ethogram outlined by Chen *et al.* [27], including occurrences of wing threat, lunging, high-level fencing, charging, holding, boxing and tussling.

(c) Experience phase

Our aggression tests indicated that mature bullies display about 18 times more aggression towards focal males on average compared to their young counterparts (see results and figure 1). Hence we generated presumed winner and loser focal males by matching them with either young or mature bullies, respectively. While we were not certain that each focal male assigned to the winner treatment was indeed a winner, and that each focal male assigned to the loser treatment was indeed a loser, this merely makes our conclusions conservative as we probably included some losers with the winners and vice versa. Prior to each test, we aspirated a single 4-day-old focal hybrid male into an aggression arena, followed by immediately aspirating either a young or mature bully competitor into the arena, and left the arenas undisturbed for 4 h. This protocol for the experience phase was identical in each of the following four experiments.

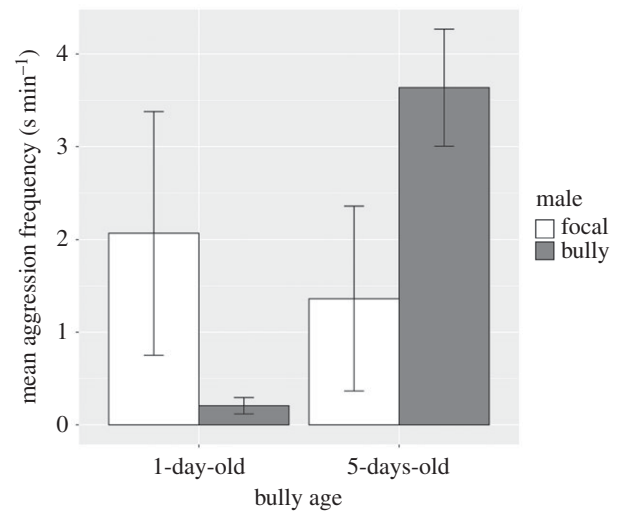


Figure 1. The mean frequency of aggressive behaviour displayed between pairs consisting of a wild-type male (focal) and either a young (1-day-old) or mature (5-day-old) hyperaggressive male (bully). The error bars represent 1 standard error of each sample. $n = 46$ pairs of males (one focal and one bully).

(d) Assay 1: pre-copulatory choice test

At the end of the 4 h experience phase, we removed the bully males from each arena and introduced a new 4-day-old competitor IV male and a 4-day-old IV female to each focal male (figure 2a). The presence of a competitor meant that mating outcomes not only depended on the attractiveness of the focal male but also on male–male interactions including aggression and courtship interference [15]. Observers blind to the focal males’ experience scanned each arena until one of the males successfully mated (or for 90 min if no mating occurred). We replicated the entire protocol across 14 days of identical sessions using 28 hybrid lines, where we tested a single male from each hybrid line and treatment combination ($n = 56$ trials per day) except in the case of missing trials. Missing trials included cases where we failed to collect sufficient numbers of males from a given hybrid ($n = 103$), or if mating did not occur in the test ($n = 12$). Hence our final sample size was 333 winners and 336 losers.

(e) Assay 2: pre-copulatory no-choice test

At the end of the 4 h experience phase, we removed the bully males from each arena and introduced a 4-day-old IV female to each focal male (figure 2b). This allowed males to court females without the interference of a competitor, meaning that the mating outcomes primarily depended on the courtship behaviour and attractiveness of the focal male [28–30]. Observers blind to the focal male’s experience scanned each arena until each mating concluded (or for 90 min if no mating occurred) and recorded all mating latencies and durations. We replicated the entire protocol across 12 days of identical sessions using 28 hybrids, where we tested a single male from each hybrid and treatment combination ($n = 56$ trials per day) except in the case of missing trials. The missing trials included insufficient numbers of males ($n = 132$) and test trials without matings ($n = 6$). Thus, our final sample size was 258 winners and 276 losers.

(f) Assays 3 and 4: female fecundity after a single mating, and ejaculate competitive ability (P1 and P2 post-copulatory success)

We conducted two experiments to assess the post-copulatory success of winner and loser focal males that mate with a

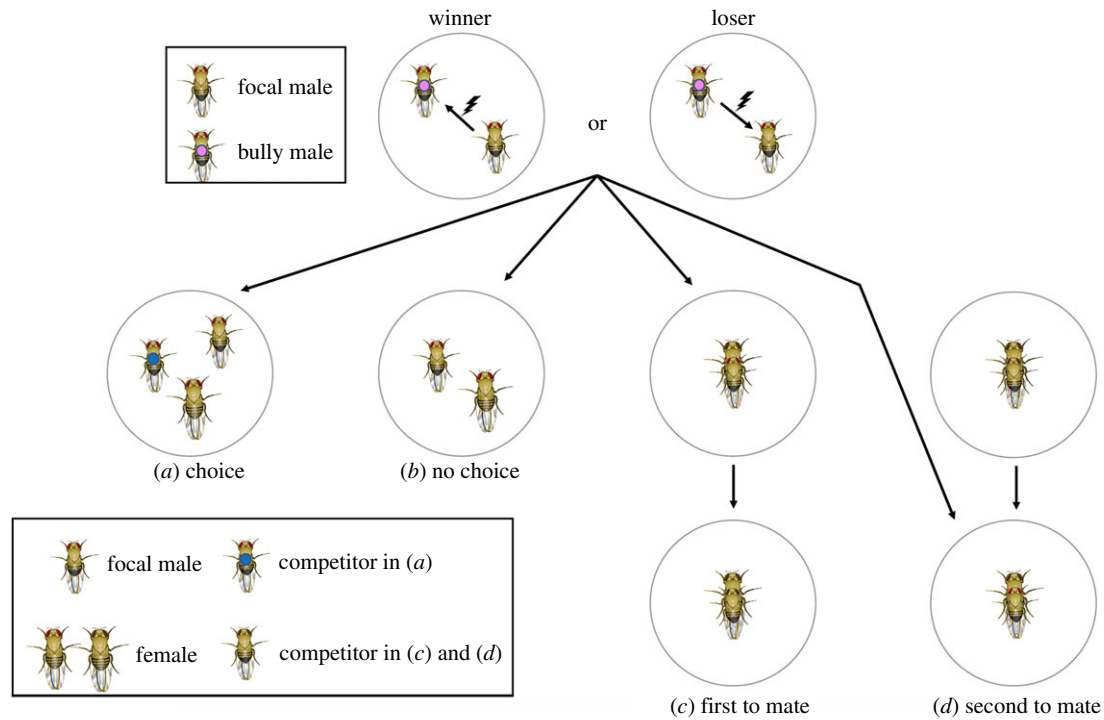


Figure 2. An illustration of the four experiments. In the first phase of all experiments, we placed each focal male inside an arena with either a docile or hyper-aggressive competitor, generating winner and loser effects, respectively. We then placed each winner or loser focal male in an arena (a) with a new competitor and a female (choice), (b) with only a female (no-choice), (c) as the first male to mate (P1) with a female or (d) as the second male to mate (P2) with a female. Note the red eyes of focal males, and brown eyes of females and competitor males in (c) and (d). (Online version in colour.)

female either first (P1) or second (P2). In the first experiment, we removed the bully males from each arena after the 4 h experience phase and introduced a 4-day-old IV-bw female to each focal male (figure 2c). We kept the focal males in the same arenas because transferring them to a new setting could reduce the winner and loser effects either merely due to the disturbance [31] or because a new site implies no or novel competitors [32]. An observer blind to focal males' experience recorded the latency and duration of each mating. At the end of each mating, we discarded the focal males, aspirated all females into individual food vials with live yeast, and placed them in the environmental chamber. On the following morning, we moved the females from the vials into new aggression arenas and returned the vials to the chamber. Two weeks later, we counted the number of offspring in each vial. This allowed us to compare female fecundity after a single mating with either winners or losers.

After adding the once-mated females into new aggression arenas, we introduced into each arena a 4-day-old IV-bw male. An observer blind to fly treatment scanned each arena until mating concluded (or for 4 h if no mating occurred) and recorded the mating latency and duration. We discarded females that did not remate ($n = 86$ and 77 in the winner and loser treatments, respectively), placed remated females into fresh vials with live yeast and housed them in the environmental chamber for egg laying over 24 h. We then discarded the females. Two weeks later, we counted the offspring fathered by focal and IV-bw males, which had red and brown eyes, respectively. We tested a random subset of six hybrids (from the original 28) and conducted three replicates. Each replicate of 120 trials consisted of testing 10 males of each of the six hybrids and two treatments. The missing trials included insufficient numbers of males ($n = 23$) and test trials without rematings ($n = 163$). Hence our final sample size of females that remated included 89 winners and 85 losers.

In the second experiment, in which we assessed the paternity success of focal males that mate with a female second (P2), we used a similar protocol as in the previous experiment except that we reversed the mating order of the focal and IV-bw

males. That is, the focal males had 4 h experience with either young or mature bullies and then were allowed to mate with females mated on the previous day to IV-bw males (figure 2d). Here, after accounting for insufficient numbers of hybrids ($n = 22$) and trials without remating (79 winners 104 losers), our final sample size included 90 winners and 65 losers.

(g) Statistical analysis

We conducted all data analyses using R v. 3.4.2 [33]. In the aggression test, the aggression frequency data were analysed using a generalized linear model with experience treatment as a main effect, and the percentage of flies data were analysed with a chi-square test. Data collected from all other experiments were analysed using generalized linear mixed models (GLMMs), created using the *lme4* package [34]. In the choice and paternity tests, we used a binomial response variable (the focal male did or did not mate), and data collected from the no-choice and fecundity test were analysed using a Gaussian response variable (mating latency and mating duration). The models included focal male experience treatment as a fixed effect, and the hybrid, the experience treatment crossed with the hybrid, and day of testing as random effects. Thus, variance in hybrid represents genetic variation in the phenotype measured, and variance in the interaction between experience treatment and hybrid represents genetic variation in the plasticity of that phenotype. In cases where our data were over-dispersed, we added an observation-level random effect [35].

We calculated the significance of the fixed effects using a log-likelihood ratio χ^2 test from the *Anova* function in the *car* package [36]. For the random effects (and their interactions), we used the *bootMer* function to calculate the 95% confidence intervals based on 1000 bootstrap samples, and the significance of each variance component using a permutation test approach [37]. This involved comparing the magnitude of our models' variance components to the distribution of 10 000 variance components that were determined from a randomized set of the experimental data.

3. Results

(a) Aggression tests

On average, mature bully males were 17.6 times more aggressive than young bullies ($\chi^2 = 28.7$, d.f. = 1, $p = 8.61 \times 10^{-8}$; figure 1). Overall, mature bullies were more aggressive than their focal competitors in 82.6% of the trials (19/23), while young bullies were more aggressive than their focal competitors in only 17.4% of the trials (4/23) ($\chi^2 = 19.6$, d.f. = 1, $p = 0.0001$; figure 1).

(b) Assay 1: pre-copulatory choice test

Fighting experience had a significant effect on the subsequent mating success of focal males competing against a novel, inexperienced male. On average, winners were successful in mating in 0.58 of the trials, and losers in 0.48 of the trials ($\chi^2 = 7.57$, d.f. = 1, $p = 0.006$; figure 3a). The effects of hybrid, hybrid by experience interaction and day were not significant (figure 3a and table 1).

(c) Assay 2: pre-copulatory no-choice test

Winners in the no-choice test were about 1.5 times faster to mate than losers ($\chi^2 = 16.464$, d.f. = 1, $p = 4.959 \times 10^{-5}$; figure 3b). The effect of hybrid was marginally significant, but the hybrid by experience interaction and day were not significant (figure 3b and table 1). Winners also had significantly shorter mating durations than losers ($\chi^2 = 32.879$, d.f. = 1, $p = 9.807 \times 10^{-9}$; figure 3c). While the effects of hybrid and day were significant, the hybrid by experience interaction was not (figure 3c and table 1).

(d) Assay 3: single-mating fecundity and sperm defensive ability (P1 paternity success)

Winners sired significantly fewer offspring than losers after a single mating with virgin females ($\chi^2 = 9.913$, d.f. = 1, $p = 0.00164$; figure 4a). The hybrid and the interaction between hybrid and treatment were not significant, but the day of testing was significant (figure 4a and table 2).

Fighting experience also had a significant effect on the paternity success of focal males mated to females first (P1), with winners having 8.4% less paternity on average compared to losers ($\chi^2 = 9.575$, d.f. = 1, $p = 0.00197$; figure 4b). Hybrid, hybrid by experience interaction and day of testing were not significant (figure 4b and table 2).

(e) Assay 4: sperm offensive ability (P2 paternity success)

Winners and losers had a similar paternity success when they were the second mating males ($\chi^2 = 0.346$, d.f. = 1, $p = 0.557$; figure 4c). While the effect of hybrid was significant, the effect of the interaction between hybrid and treatment and day were not (figure 4c and table 2).

4. Discussion

Our results indicated that, on average, (i) winners performed better than losers in pre-copulatory contests, (ii) losers performed better than winners in post-copulatory contests and (iii) the expression of some of this plasticity may be due to

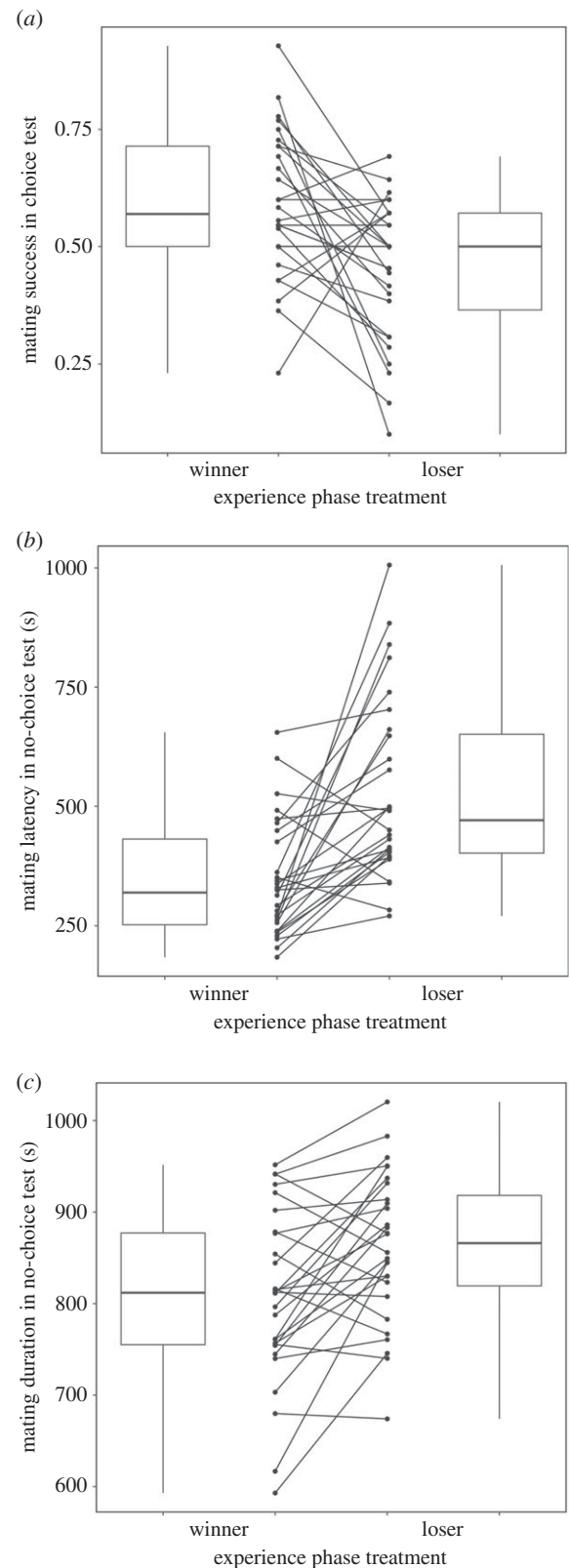


Figure 3. Effects of previous fighting experience on pre-copulatory mating success in males. The boxplots depict the distribution of the entire dataset of each experience treatment. The boxes contain the middle 50% of data (interquartile range, IQR), and the horizontal lines represent the medians. The whiskers above and below each box represent values within $\pm 1.5 \times$ the IQR. The reaction norm plot in the centre of each panel depicts the change in the calculated mean of each hybrid across the two experience treatments. (a) The mating success of winner and loser focal males in mate choice trials each involving a focal male, a competitor male and a female. (b) The mating latencies of winner and loser focal males in no choice trials each involving a focal male and a female. (c) The mating durations of winner and loser focal males in the no-choice trials.

Table 1. Variance components, standard deviation, 95% confidence intervals and *p*-values estimated using a GLMM fit by maximum-likelihood (Laplace approximation) for the reproductive success of hybrid males in pre-copulatory contexts. Males from different genetic backgrounds were randomly assigned as winners or losers.

fitness	source of variance	variance (s.d.)	bootstrapped upper and lower 95% CI	percentage of variance explained	<i>p</i> -value
mating success	hybrid	0.038 (0.195)	0.248, 0	3.08	0.32
	hybrid \times experience	0.189 (0.435)	0.674, 0	15.4	0.127
	day	0.004 (0.064)	0.03, -0.381	0.32	0.787
	residual	1			
mating latency	hybrid	13803 (117.49)	44375, 11.3	5.68	0.052
	hybrid \times experience	10090 (100.45)	33454, 0	4.15	0.247
	day	8270 (90.94)	322.6, -36161.5	3.41	0.918
	residual	210698 (459.02)			
mating duration	hybrid	4323.18 (65.75)	8074.3, 1.1	17.4	<0.001
	hybrid \times experience	98.35 (9.92)	1756.3, 0.361	0.4	0.617
	day	1527.16 (39.08)	2018.7, -1551.8	6.14	0.001
	residual	18936.6 (137.61)			

natural genetic variation present in the focal population. Thus, the outcomes of aggressive interactions can have important consequences for the evolution of sexually selected traits by influencing the reproductive success of winners and losers. Here, we used randomly assigned winners and losers and found that the outcome of a variety of reproductive competitions can be significantly affected by the previous fighting experience. Previous studies have documented that the experience of winning or losing can influence the expression of pre- [7] and post-copulatory reproductive traits [12]. We add to these findings by documenting the differential effects of winning and losing on pre- and post-copulatory success. We also found segregating genetic variation underlying some of our measures of male fitness. Additionally, our methodology ensures that our results are due to changes in the expression of reproductive traits (phenotypic plasticity) resulting from the outcomes of social experience, as opposed to intrinsic differences between winners and losers. This is an important difference that many previous studies have failed to distinguish due to selection bias [1,2].

When looking at the effect of fighting experience on pre-copulatory success, we found that winners significantly outperformed losers in both the choice (two males) and no-choice (one male) tests. In the choice tests, it is likely that winners experienced prototypical winner effects related to increased aggression and fighting ability [38]. When two males are placed in an arena with a single female, the mating outcome may be influenced by the aggressive interactions between the males through courtship interference [15] or via female choice [30]. If winners are more aggressive than losers, this can explain the observed difference in mating success between the two treatments through increased courtship interference. Future studies should continue to attempt and untangle the relative contribution of male–male competition and female choice in these types of interactions [15].

The increased success of winners in our no choice tests may be explained by differences in male attractiveness and courtship behaviour. Shackleton *et al.* [28] argued that

measuring mating latency is a reliable indicator of male attractiveness, so one possibility is that this difference may be explained by winners being more attractive than losers. Previous studies have found conflicting results regarding social dominance and attractiveness. In some cases, winners may be preferred when fighting ability can signal and/or covary with good genes, where in other cases losers are preferred when fighting ability may signal a cost to females via an increased potential for sexual harassment [39,40]. However, in species with intense sexual conflict, mating with sexually coercive males can also provide indirect benefits to females via ‘sexy sons’ and thus harmful males are thought to be preferred by females in *D. melanogaster* [41] (but see [42]). Assuming winners are more harmful in a pre-copulatory context [39], the sexy sons hypothesis is consistent with our results. Future studies should quantify differences in male pre-copulatory harassment between winners and losers to determine the associations between attractiveness, pre-copulatory male harm and winner–loser effects. Another possibility is that this difference may be explained by differences in courtship behaviour between winners and losers. In *Drosophila*, Kim *et al.* [43] found that losers take around two times as long to initiate courtship when placed into a chamber with a virgin female, which may explain the difference that we observed in mating latency. Another interesting possibility may involve differences in cuticular hydrocarbon expression between winners and losers, which can significantly influence the outcomes of mating interactions [44]. In *Drosophila*, an individual’s expression of these hydrocarbons is highly sensitive to its sociosexual environment [45,46]. Future studies should attempt to quantify traits that are known to influence attractiveness, such as courtship ability and cuticular hydrocarbon profiles, and compare them between winners and losers.

When looking at the effect of fighting experience on post-copulatory success, we found that losers significantly outperformed winners in the fecundity and sperm defence tests, but not in the sperm offence tests. These results are

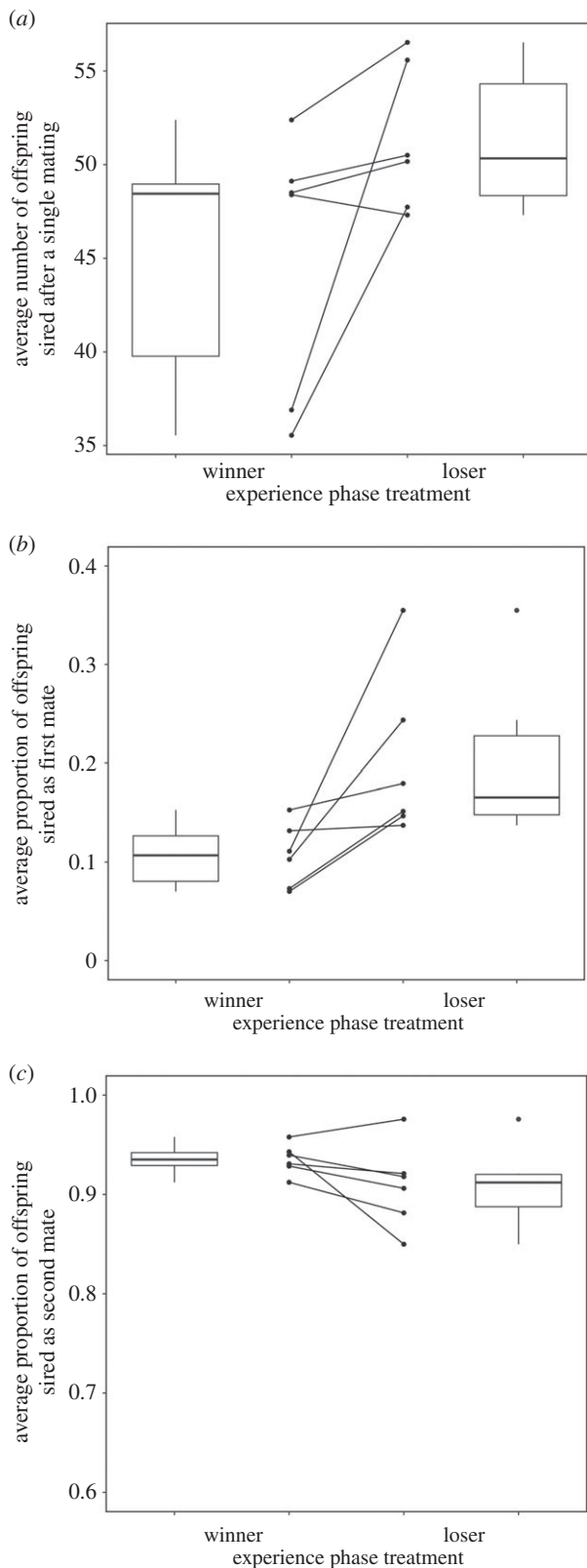


Figure 4. Effects of previous fighting experience on post-copulatory mating success in males. The boxes contain the middle 50% of data (interquartile range, IQR), and the horizontal lines represent the medians. The whiskers above and below each box represent values within $\pm 1.5 \times$ the IQR, and any values beyond this are outliers represented by closed circles. The reaction norm plot in the centre of each panel depicts the change in the calculated mean of each hybrid across the two experience treatments. (a) Male effect on a single day of female fecundity after a single mating. (b) Male success in sperm defence assay (focal first male to mate), where success is defined as the proportion of offspring sired. (c) Male success in sperm offence assay (focal second to mate), where success is defined as the proportion of offspring sired.

mostly consistent with studies that predict that dominant males trade-off by investing less resources into post-copulatory mechanisms due to a potentially greater number of future mating opportunities (and vice versa for losers) [47]. As mating duration is positively correlated with investment into the transfer of accessory gland proteins (Acps) [17], the increased duration of mating displayed by losers in our experiments may suggest a greater transfer of Acps. In the case of fecundity, losers may transfer larger quantities of Acps in their ejaculate to maximize the number of offspring they produce, and in the case of sperm defence, to maximize their share of paternity after potential rematings [48]. In a variety of species, it is well understood that extrinsic sociosexual factors can cause rapid changes in male mating strategies [11,49,50], but we are the first to demonstrate that winner and loser effects play an important role. The fact that losers had a higher share of paternity and produced more offspring when they were first to mate offers insight into how this plasticity may be selected for. Given that the transfer of Acps has toxic-side effects that reduce the fecundity and lifespan of females [51], future studies should investigate the consequences of winner–loser effects for female fitness. Paired with the findings of our first two assays, it may be that winners are more harmful in a pre-copulatory context, while losers inflict more harm via post-copulatory mechanisms.

As sperm defensive and offensive abilities appear to be phenotypically uncorrelated in *D. melanogaster* [52], it is not surprising that we found no difference in sperm offensive ability between winners and losers, despite our detected difference in sperm defensive ability. One possible explanation is that the decreased sperm competitive ability of males carrying the *bw* mutation reduced the resolution of our results. This disadvantage has been identified by previous studies [53] and is clear in our results, as the average P2 success was 84.7% for males with the *bw* mutation versus 92.4% for focal males. Additionally, given the strength of last male sperm precedence in fruit flies [54], it would be more advantageous for subordinate males to focus on improving their sperm defence as opposed to offence. This hypothesis is consistent with evidence for a positive correlation between sperm defence success and male-induced harm, but no correlation between sperm offence success and male-induced harm [54]. Future studies should attempt to quantify the Acp profiles and sperm characteristics of winners and losers to untangle the mechanisms underlying this plasticity.

Finally, it appears that some of the differences we observed in various metrics of male reproductive success were due to natural genetic variation present in the population. Although our statistical models only detected significant genetic variation in mating duration and sperm offensive ability, a sizeable proportion of the variances for each of the phenotypes we measured were explained by hybrid (tables 1 and 2). As other experiments that focused on detecting genetic variation in traits such as sperm competitive ability have identified significant variation [55], it is likely that we did not have enough power to detect it in our models. Thus, it is still important to note the potential importance and implications of genetic variation for the expression and plasticity of these traits. Similarly, although we did not detect significant variation in the interactions between winner–loser treatment and genotype, our models indicate that a sizeable proportion of the observed variance

Table 2. Variance components, standard deviation, 95% confidence intervals and *p*-values estimated using a GLMM fit by maximum-likelihood (Laplace approximation) for the reproductive success of hybrid males in post-copulatory contexts. Males from different genetic backgrounds were randomly assigned as winners or losers.

fitness	source of variance	variance (s.d.)	bootstrapped upper and lower 95% CI	percentage of variance explained	<i>p</i> -value
fecundity	hybrid	1.052 (1.026)	13.949, 0	0.5	0.369
	hybrid × experience	3.139 (1.772)	24.561, 4.3×10^{-14}	1.5	0.393
	day	13.487 (3.672)	49.971, 0	6.69	0.014
	residual	183.869 (13.560)			
P1 paternity	individual	1.76 (1.328)	2.218, 1.188	58.7	0.97
	hybrid	0.055 (0.234)	0.298, 11.3	1.8	0.198
	hybrid × experience	0.181 (0.344)	0.619, 1.13×10^{-11}	6	0.225
	day	1.6×10^{-9} (4×10^{-5})	0.004, −0.404	0	0.837
	residual	1			
P2 paternity	individual	0.971 (0.985)	1.399, 0.668	40	0.994
	hybrid	0.251 (0.501)	0.715, 5.9×10^{-12}	10.3	0.007
	hybrid × experience	0.154 (0.391)	0.69, 4×10^{-11}	6.3	0.096
	day	0.051 (0.226)	0.002, −0.599	2.1	0.973
	residual	1			

was due to differences between hybrids, and the interaction between hybrid and treatment. This means that the magnitude and direction of winner–loser effects on the phenotypes we measured depend on individual genotype (figures 3 and 4). This may explain the maintenance of genetic variation for different male reproductive strategies [56]. If some males improve their reproductive success in a given context as winners, but others improve as losers, then different reproductive strategies can be maintained, even only if one is favoured by female choice.

Taken together, our results indicate that some of the variation we observe in male mating strategies is due to the experience of winning or losing in male–male interactions. The fact that winners appear to focus on pre-copulatory strategies, while losers focus on post-copulatory strategies may represent a trade-off between reproductive strategies that is mediated by winner–loser effects. This variation in mating strategies can influence male reproductive success, and thus the strength and direction of evolution via sexual selection. If winners and losers consistently benefit from investing into pre- and post-copulatory strategies, respectively, then this plasticity may be selected for in many species. Given the ubiquity of winner–loser effects throughout the animal kingdom [1], this plasticity may also explain the maintenance

of variation in male reproductive strategies in many species. These results also highlight the importance of considering multiple metrics of fitness when exploring the ultimate causes of phenotypic variation. Finally, our results provide the groundwork for understanding the biological bases of persisting states of moods associated with winning and losing and their fitness consequences in many animals including humans.

Data accessibility. Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.2hg1785> [57].

Authors' contributions. D.C.S.F. carried out the experiments. Both authors designed the experiments, contributed to the data analysis, writing, and editing process of the manuscript. Both authors approve of the final manuscript submission and hold accountability for the accuracy and integrity of its contents.

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