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**Loser-effect duration evolves independently of fighting ability.**

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Abstract

Winning or losing contests can impact subsequent competitive behaviour and the duration of these effects can be prolonged. While it is clear effects depend on social and developmental environments, the extent to which they are heritable, and hence evolvable, is less clear and remains untested. Furthermore, theory predicts that winner and loser effects should evolve independently of actual fighting ability, but again tests of this prediction are limited. Here we used artificial selection on replicated beetle populations to show that the duration of loser effects can evolve, with a realized heritability of about 17%. We also find that naïve fighting ability does not co-evolve with reductions in the duration of the loser effect. We discuss the implications of these findings and how they corroborate theoretical predictions.

Key words: Winner effect, loser effect, fighting, contest, realised heritability
Introduction

In many animals aggressive contests occur for limited resources like territories, food and mates (1). Additionally, individuals frequently engage in repeated contests, and previous fighting experience often influences current contest outcomes (2). Thus prior winning often increases the probability of winning subsequent contests, and prior losing decreases the probability, phenomena known as winner and loser effects respectively. While the precise underlying causes of winner/loser effects are often unclear, they are assumed to have some genetic underpinnings (reviewed in 3). However, despite the widespread occurrence of winner/loser effects (2, 3), this claim is rarely tested – in fact we could find no examples where explicit tests of a genetic basis to winner/loser effects had been undertaken. Additionally, although we expect genetic variation to underpin behavioural phenotypes (4), this might not always be the case (5).

One explanation for winner and loser effects is that prior experience shapes future contests by providing contestants information about their relative resource-holding potential (RHP) or fighting ability (6), and two non-mutually exclusive hypotheses for the effects have been proposed (3). Individuals either
gain information on their own RHP (a self-assessment mechanism) or winning and losing produces status-related cues that affect the assessment of subsequent opponents (a social-cue mechanism). With a social-cue mechanism, individuals are predicted to detect previous winning or losing by their opponents from visible or chemical ‘cues’ emitted by them, and should adjust self-behaviors based on opponent’s previous experience (3). Social-cues include signs of exhaustion or injuries (7), and odors (8). Both hypotheses require there to be variation in fighting ability in the population so that there is value in working out who to fight and who not to fight (9). A typical example of self-assessment is learning through prior fighting (10, 11). Here, individuals adjust their behavior based on their previous experience (e.g., 12-14), and there is abundant evidence that individuals vary in their behavioural adjustments, including in contest duration (2) and the type of adjustments employed (14-16). Additionally, variation in behavioral adjustment may be underpinned by differences in perception (10) and learning ability (11), and this variation can be related to behavioral syndromes or personality (17). Finally, although the evolution of winner and loser effects can be inferred from such among-individual differences (3, 11), direct evidence for genetic variation and responses to selection of winner-loser effects appears to be
lacking. This may be because these effects arise from experience, effectively the environment. But of course the environment is responsible for all manner of gene expression variation that generates physiological changes in an individual, and any genetic variation in gene expression (e.g. 18) will mean genetic variation for winner/loser effects. Thus genetics will also be important (2).

Here we only focused on loser effects and their duration. This is primarily because theory suggests loser effects can evolve without corresponding winner effects, while the reverse is not true (3, 19). This loser only evolution should occur when the costs of fighting (C: the rate of increase in costs of over-estimating RHP in terms of heightened risk of getting into and losing escalated fights) are moderate and the fitness benefits of dominance (V: relative fitness of dominant individuals) are substantial (e.g. V > C > 0), a pattern reported for several taxa (e.g., 20-22). Furthermore, although experience effects are generally short-lived, as noted above, variable durations are found within and across taxa (reviewed in 3). For example, effects can persist from 10 minutes to 10 days (e.g. 23, 24), and although there is limited evidence for the intra-specific variation in loser effects (2), variation has been found in the cricket *Gryllus bimaculatus* (23, 25-27). Variation in the duration
of effects is thought to be influenced by the frequency of social interactions and population density (22, 28), as well as the costs and benefits of fighting (2), which all implies that these effects can evolve. Interestingly, effects may be due to perception only. That is, absolute fighting ability need not reflect the duration of loser effects and *vice versa*. So loser effects could potentially evolve without affecting fighting ability, although this remains to be demonstrated experimentally.

Broad-horned flour beetles (*Gnatocerus cornutus*) are increasingly well studied, especially with respect to their fighting behaviour and its consequences (e.g. 14, 29-36). Males freely engage in combat for access to females (14) and experience a loser effect when they are beaten in these fights. The loser effect lasts for about four days, during which time fewer than 25% of losers will engage in combat (75% of losers will not fight), and there is no apparent decay of the effect during that four-day period (14). Rather than fighting, losing males tend to disperse to new territories (which may or may not contain other males) and increase their investment in sperm production (14, 35). It should be emphasized that there is no modulation of male behavior due to winning (i.e. winners are not different from naïve males), which is consistent with theoretical predictions that loser effects can
evolve alone (3, 19). Here we investigated whether the duration of the loser effect could evolve through artificial selection in experimental populations of *G. cornutus*. Any response to selection would then facilitate estimating the heritability of the response duration and enable testing for correlated evolution of male fighting behaviour. Furthermore, demonstrating such evolutionary responsiveness would establish the broad-horned flour beetle system as a model for explicit testing of theoretical predictions about the conditions under which pure loser effects are expected to evolve (19).

**Materials and Methods**

The *G. cornutus* beetle culture originated from adults collected in Miyazaki City (31° 54’N, 131°25’E), Japan, and has been maintained in the laboratory of the National Food Research Institute, Japan, for ~50 years on whole meal enriched with yeast. The stock contains 1500–2000 beetles per generation. This beetle is a stored product pest, and thus, the laboratory conditions very closely mimic what have become natural conditions over the last 4500 years (37). All rearing and subsequent experimentation was conducted in a chamber maintained at 25°C, 60% relative humidity and with a photoperiod cycle of 14:10h light/dark.
To obtain virgin adults for experiments, one final instar larva was placed in each well of a 24-well tissue culture plate with 1g of food (Cellstar; Greiner Bio-One, Frickenhausen, Germany) (14, 32). Individuals were placed in the wells immediately after eclosion, and did not interact with conspecifics until the start of the experiments. Thus, we ensured that animals were virgin and had no previous fighting experience. Adults 15-20 days old (after final eclosion) were used for the experiments (For a more detailed description of the stock culture see references 14, 32). The body size (prothorax width: 14, 32, 38) of each experimental individual was measured (±0.01 mm), using a dissecting microscopic monitoring system (VM-60; Olympus, Tokyo, Japan)(see 32 for landmarks).

Identifying losers

Following established protocols (14), adult males with no fighting experience were collected from the stock culture (collected as final instar larvae and housed alone until adulthood). To control for the effect of body size on fighting success, males were paired so that the difference in body size between contestants was less than 0.01 mm thus competitors differed by in size by less than 2% (14). Pairs were placed on filter-paper (17 mm diameter) in a plastic container (17 mm diameter,
20 mm high) and allowed to interact (and fight) for one hour - previous work has shown that male fights occur in almost all trials when staged in this manner (14).

Males that pushed opponents and chased them were denoted the winner (14).

Losers (L-males) were those that retreated from the winner. For a more detailed description of the methods, see (14). Subsequently, each L-male was placed in one well of a 24-well tissue culture plate with food (1 g), as described above, until testing for the selection and control populations. These focal males were marked with white or pink spots [Mitsubishi Paint-Marker] on their elytra; in half of the trials, focal L males were white, and in half of the trials, focal L males were pink.

Selection protocol

As shown previously (14), loser effects last about 4 days, with no apparent decay in the proportion of males affected during that period (and again note there is no modulation of behavior due to winning fights). Here we selected for a reduced duration of the loser effect after losing fights. We first collected males from the stock culture to manipulate the loser effect, as described above, to establish three selection and three control populations (initially with ca. 75 males/population). To investigate whether the loser effect influenced the outcome of a subsequent fight,
each loser male (males that lost initial fights) was matched with an opponent male

collected from the stock culture (tester male), at 4 days after first fight losses. The
tester males had no fighting experience in these or other experiments. Contestants
were matched for body size (as above) and outcomes were assessed as above. We
then selected the 12 losers that won these second fights (i.e., males that had not
modulated their behavior until day 4 due to their previous losing experience) as
sires of the reduced loser-effect-duration populations (RLE Populations). To
propagate control populations (C Population) 12 random (with respect to their
fighting behaviour in these second bouts) (previous) losers where selected to act
as sire. That is, control males had also lost initial fights, but we did not take their
subsequent win/lose status into account when choosing them as sires. The 12
males/population were randomly divided into four groups (three males in each),
and each group was placed in a plastic cup (7 cm diameter, 2.5 cm height) with 20
g of medium and three females collected from the stock culture. Groups were
maintained this way for 2 months with males able to mate with females and
females were allowed to lay eggs in each group, until final instar larvae were
obtained (38). Final instar larvae were collected (as above) to obtain the adults for
subsequent generations. When the adults reached 10–15 days old, 144
males/population were randomly collected and 72 male pairs/population were matched within each population and tested again as above. We then took losers from these fights and selected the 12 losers that won second fights against tester males (4 days later) to propagate RLE Populations, and randomly selected 12 previous losers regardless of their winner or loser status in second fights to propagate controls (C Populations). Females were randomly chosen as dams from within each experimental population. This regime continued for 10 generations.

We randomly collected 50 males from each experimental population at generation 5 and 10 and examined whether the duration of the loser effect had decayed at day 4; we examined whether males were attacked first by or lost fights to tester males, again noting that usually almost all losers will not fight so not initiating attacks is a measure of loser effect duration (14). Winning or losing a fight provides an estimate of fighting ability.

To compare population rates of attacking first and losing fights, we applied a generalized linear model (GLM) with a binomial distribution, a logit-link function, and overdispersion test. Replicate (population) was nested within selection regime [RLE = reduced loser effect duration and C = control (no artificial selection on loser]
effect duration). Losing (losing=1, winning=0) and attacks (attacked=1, attacking=0) were the response variables. All model assumptions were met. All statistical analyses were carried out using JMP 7 (39). The realized heritabilities were calculated according to the liability model (40) as cumulative response to selection divided by cumulative selection differential (also see 41).

In addition to testing for an impact of selection on loser effect at day 4, we also tested effects of losing on males at days 1-5 after they lost their initial fights (using the methods previously described, with different males used for each day – each male only fought twice). On each day after initial losses, 20 losers (per day) were observed per population (n = 600 in total), in which the experimental losers competed against tester males from the stock culture. Population rates of being attacked first or losing to by tester males were compared using GLMs with a binomial distribution, a logit-link function, and overdispersion test. Replicate was again nested within selection regime (RLE and C), and this and test time (day after initial loss 1-5) were used as the explanatory variables. Losing (losing=1, winning=0) and attacks (attacked=1, attacking=0) were the response variables. When significant interaction terms (selection regime × day) were observed, as a
post-hoc test, we compared population rate at each day using GLMs with a binomial distribution, a logit-link function, and overdispersion test. Replicate was nested within selection regime (RLE and C), and this was used as the explanatory variable. We note here that when we used model selection (Table S1) inferences were unchanged.

**Correlated responses in fighting success**

At generation 10 adults were also collected to assess the fighting success of naïve males as a correlated response to selection on loser effect duration. Males from each of the six experimental populations (RLE and C) were used to assess fighting success when they had experienced no previous fighting – their first fights when they were naïve. We observed 30 contests per population (n = 180 in total) in which focal experimental males competed against a tester male (as described above). Trials were then continuously observed until fight outcomes could be scored. Population rates of being attacked by and losing to tester males were compared using GLM with a binomial distribution, a logit-link function, and overdispersion test. Replicate was nested within selection regime (RLE and C) and this was used as the explanatory variable. Losing (losing=1, winning=0) and
attacks (attracted=1, attack=0) were the response variables.

Correlated response in body size

Thirty males were also randomly chosen from each of the six experimental populations (RLE and C) at generation 10 and were used to assess body size (prothorax width). Body size was compared using GLM with a normal distribution and an identity-link function. Replicate was nested within selection regime (RLE and C), which was the primary explanatory variable.

Results

The proportion of previous losing males that lost focal fights to tester males at 4 days after initial losses showed a clear direct response to selection (figure 1), with a steady divergence between selection and control populations (Generation 5: selection, d. f. = 1, \( \chi^2 = 21.51, p < 0.001 \); replicate (within selection), d. f. = 4, \( \chi^2 = 4.27, p = 0.31 \). Generation 10: selection, d. f. = 1, \( \chi^2 = 47.87, p < 0.001 \); replicate (within selection), d. f. = 4, \( \chi^2 = 1.11, p = 0.89 \); figures 1 and 2ac). After 10 generations of selection, the proportion of males losing their second fights 4 days after initial losses in the RLE populations had fallen to around 50%, whereas it was
always higher than 80% in control populations. Similar results were observed in the proportion of previous losing males that were first attacked by tester males at 4 days after initial losses (Generation 5: selection, d. f. = 1, $\chi^2 = 15.52$, $p < 0.001$; replicate (within selection), d. f. = 4, $\chi^2 = 2.12$, $p = 0.71$. Generation 10: selection, d. f. = 1, $\chi^2 = 27.69$, $p < 0.001$; replicate (within selection), d. f. = 4, $\chi^2 = 1.18$, $p = 0.88$; figure 2bd). However, males of the RLE populations had not become completely immune to losing fights. In the first three days after initial fight loss, the RLE males behaved much the same as control males losing second contests about 85% of the time (figure 3a), while the control males did not fall to the day 4 levels of selection male success (50% of fights won) until 5 days after initial losses (figure 3a). Similar results were found when we compared which males attacked first, with RLE males tending to become more aggressive only at day 4 and control males not achieving this level of aggression until day 5 after initial losses (figure 3b). This all indicates that loser effect decayed after 3 days in the RLE populations, and after 4 days in control populations. Thus, we found a significant difference in the duration of loser effects between experimental treatments.

This rapid response to the selection indicated heritable variation in the effects
losing has on males. Realized heritabilities were significantly different from zero for all RLE Populations \( h^2 (\pm SE) \): RLE I, 0.188 (0.015); RLE II, 0.179 (0.021); RLE III: 0.161 (0.024). All \( p < 0.001 \), with 16-19% of the variation in the duration of the loser effect estimated to be due to additive gene action.

Selection on the duration of the loser effect did not affect male fighting success and likelihood of initiating attacks on rivals when males had no previous fighting experience. Naïve males from the selection populations attacked as much and won/lost as much in their initial fights as males from control populations (Initiate Attacks – RLE, 0.53, 0.50, 0.57: C, 0.47, 0.43, 0.53: selection, d. f. = 1, \( \chi^2 = 0.56, p = 0.46 \); replicate (within selection), d. f. = 4, \( \chi^2 = 0.89, p = 0.93 \). Fights Lost – RLE, 0.43, 0.53, 0.53: C, 0.50, 0.57, 0.47: selection, d. f. = 1, \( \chi^2 = 0.02, p = 0.88 \), replicate (within selection), d. f. = 4, \( \chi^2 = 1.43, p = 0.84 \). Furthermore body size did not evolve as a correlated response to selection on loser effect duration (Body size (mm\( \pm \) SE): RLE, 1.214 (0.006), 1.222 (0.006), 1.214 (0.005): C, 1.217 (0.007), 1.207 (0.005), 1.208 (0.007): selection, d. f. = 1, \( \chi^2 = 1.42, p = 0.23 \), replicate (within selection), d. f. = 4, \( \chi^2 = 2.84, p = 0.58 \).
Discussion

Our major findings here were that the duration of loser effects can evolve, with narrow sense heritabilities of about 17%, and furthermore, the evolved, reduced duration of the loser effect was not simply due to a general loss of the effect. Additionally there appeared to be no general change in fighting ability (as measured by fighting success in first fights) or body size that evolved as correlated responses to selection on loser effect duration. We discuss these findings further below.

Perhaps the most interesting finding was that in the populations that evolved shorter loser-effect durations, fighting success in contests between naïve animals did not evolve – there was no difference in success rates between control and experimental populations. This suggests that actual fighting ability in these dyadic contests had not evolved in response to our selection, but clearly there was a reduction in the effects losing had on subsequent behaviours in the experimental populations. This contrasts somewhat with crickets were winning is associated with a broader range of fighting tactics (42), but the fact that beetle populations evolving reduced impacts of losing had not changed their fighting success (% naïve
wins) only serves to highlight the differences between fighting ability and the
impacts of losing. Indeed, the fact that loser effects can evolve independently of
fighting ability establishes broad-horned flour beetle as an ideal system to test
formal theoretical predictions about when loser effects are expected to evolve by
themselves (19). That is, in testing how fighting costs and dominance benefits
affect the disconnect between loser and winner effects, and for example, testing
whether increasing variation in fighting ability within populations selects for
stronger loser effects as predicted by theory (9, 19). Future work could therefore
manipulate key parameters in different populations and quantify any concomitant
evolutionary change in loser effects.

Body size also did not evolve as a correlated response to selection, which given the
lack of change in fighting ability is arguably not surprising. Size frequently
determines RHP, and RHP should correlate with an individual’s absolute
probability of winning fights (6). However, fighting ability is also associated to
other factors like fighting skills and physical performance (43-45). Indeed, recent
work has shown that fighting ability can be linked to measureable functional traits
such as bite force (reviewed in 43), and the loser effect is associated with a
decrease in bite force in the cricket *Acheta domesticus* (44). Further studies are required to investigate precisely what determines fighting ability in *G. cornutus*, but our results suggest that the loser effect and fighting ability of naïve (with respect to fighting) males are not closely genetically linked in this species - males from populations selected for reduced duration of the loser effect did not win more initial fights than control males (nor where they larger), so it appears functional traits linked to absolute ability did not coevolve with reduced loser effects. This finding corroborates assumptions in the theoretical literature, which posit that loser (and winner) effects reflect changes in subjective estimates of the distribution of fighting abilities in the population but not changes in individual fighting abilities *per se* (9).

While experience effects are often short-lived, they vary in their durations and duration can be affected by costs and benefits of fighting and social interaction frequency (2, 22). These general inferences are mirrored in a theoretical study of *G. cornutus* fighting behaviour, which predicted that the optimal duration of the loser effect would depend on the frequency of social interactions, the mating success derived from fighting (benefit) and the decrease in longevity resulting from
fighting (cost) (28). Again, these findings all suggest effects can evolve, as we have shown here. Interestingly, the heritability of the loser effect we report is on the low side for a behaviour (46) and this probably reflects the fact that there are many links in the causal pathway generating the effect. That is, we may have selected on memory retention or metabolic rate for example, but have not directly estimated the heritability of memory or metabolism. Additionally, a number of studies have implicated biogenic amines such as octopamine or dopamine as neurochemical mechanisms of winner/loser effects (e.g. 23, 47; reviewed in 48). Thus by selecting on the duration of the loser effect we may well have altered the time course of octopamine effects, or those of an octopamine agonist. We did not test for these possible changes, and there are of course mechanisms other than these that could be involved in generating the evolutionary change we document, including a raft of other physiological and neurological processes (49, 50) that could have been altered by the artificial selection we applied. We finally note that realized heritabilites are only approximations of base-population heritabilities (40), and that there was no evolution of effects in the control lines.

The relatively low heritability also implies that, as expected, much of the variation
in the loser effect is environmental. Outcomes of direct physical fights will obviously depend on opponents and will provide reliable information enabling self-assessment of one’s own fighting ability relative to others in the population. Thus the social environment and an individual’s developmental environment must influence winner/loser effects to a large degree and thus contribute much to phenotypic variation in these effects (e.g., 51, 52).

Behavioural modulations resulting from winning fights have not been recorded in *G. cornutus* (14) even though the loser effect has a relatively long duration. This matches a general pattern of effect decay, with loser effects generally lasting longer than winner effects (2). For example, losing fights impacts sticklebacks for around 6 hours, but the winner effect has largely disappeared after about 3 hours (53). From a proximate perspective, it has been suggested that this asymmetry is a consequence of fundamental learning processes: losers may have more control over situation outcomes (i.e., they can retreat but individuals cannot determine whether a fight will occur or not as that depends on opponent behaviour) and hence links (activity-outcome) are easier to establish and remember (19, 54).
Given the methodological impacts on winner/loser effect assessment, it is important to note that individuals in our investigation were self-selecting (*sensu* 2) (i.e. we did not randomly allocate subjects to winner/loser treatments), were isolated for much of their lives and had very few encounters with competitors. Each of these factors can potentially affect individual experience (2). In our beetles, individuals are normally likely to encounter multiple rivals throughout their lives, these multiple encounters will probably result in more complex effects, with each individual experience potentially contributing to cumulative effects on future contest outcomes (e.g. 55, 56).

Fighting experience effects can also impact multiple behaviours and ecological processes that we did not assess here (e.g. 57, 58). Indeed, the loser effect can impact various reproductive and dispersal strategy in *G. cornutus* beyond the fighting outcome itself (14, 35). Similarly, theory predicts that many factors can influence the strength of loser effects, including age and experience (e.g. 59, reviewed in 9), and many of these are untested in flour beetles. Furthermore, the effects of male experience could impact female reproductive behaviours in this beetle. There are direct fitness costs imposed on females by aggressive,
competitively superior males (60). These males are highly aggressive towards rival males (38) but also attack females (60). Thus female fitness-costs are probably side-effects of misdirected male aggression, as suggested for the dung fly, *Sepsis cynipsea* (61). Many studies have now demonstrated that highly competitive males can be harmful to females (e.g. 62-66) and in *G. cornutus*, show female mate-preference may be constrained, because although females prefer males that fight less, they frequently do not get to mate with them (33, 60).

To conclude, we used artificial selection to cause micro-evolution of the duration of the loser effect. We also found that the reduction in response duration was not associated with a change in fighting ability (as measured by the likelihood of fighting success), which supports theoretical predictions. Further investigations of these effects and on the precise neural/physiological mechanism underpinning the outcomes of our artificial selection are warranted. Our work also suggests broad-horned flour-beetles are an excellent model to explicitly test theoretical predictions about the conditions under which pure loser effects are expected to evolve.
Competing interests

We have no conflicts of interest.

Authors' contributions

KO collected data, carried out the statistical analyses, participated in the design of the study and drafted the manuscript; YO, SD and DH conceived of the study, coordinated the design of the study and drafted the manuscript. All authors gave final approval for publication.

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**Figure legends**

**Figure 1.** Responses to selection on loser-effect duration. The proportion of males with losing experience (L males) that lost subsequent fights to a tester male at 4 days after losing initial fights (our measure of loser effect duration). White circles, are the populations where we selected for reduced duration of the loser effect (Reduced Loser Effect duration: RLE). Black circles are the Control Populations (C) that were not subjected to selection on the duration of the loser effect.

**Figure 2.** The proportion of males with losing experience (L males) that lost subsequent fights to a tester male at 4 days after losing fight (our measure of loser effect duration) and L males that were attacked first by a tester male (i.e. focal males that did not initiate attacks) at 4 days after losing fight at generation 5 (a, b) and 10 (c, d). RLE populations are those where we selected for reduced duration of the loser effect (Reduced Loser Effect duration). The Control Populations (C) were not subjected to selection on the duration of the loser effect.

**Figure 3.** Loser effects at each day after losing initial fights in focal experimental males – White circles, are the populations where we selected for reduced
duration of the loser effect (Reduced Loser Effect duration: RLE). Black circles are
the Control Populations (C) that were not subjected to selection on the duration
of the loser effect. (a) is the proportion of focal males that lost subsequent fights,
and (b) is the proportion of focal males attacked first by tester males (i.e. focal
males that did not initiate attacks). There was neither an effect of selection
regime nor replication, but there was a significant interaction between selection
regime and day (Selection × Day, d. f. = 4, $\chi^2 = 30.26, p < 0.001$).