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Animal behaviour

Genetic correlation between aggressive signals and fighting

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Theoretical analyses indicate that aggressive signals should positively correlate with the signallers' willingness and abilities to fight. Few experimental studies, however, have tested this prediction. In two experiments employing distinct, ecologically realistic protocols, we quantified the association between aggressive signals and fighting in fruit fly genotypes and found high positive genetic correlations between threat and fighting (r_G = 0.80 and 0.74). Our results add to the growing body of experimental work indicating that aggressive signals have relatively high informational value.

1. Introduction

Many animals rely on aggression for access to desirable resources and mates. While such aggression has obvious potential fitness advantages, fighting may also have severe costs, which range from lost time and energy to wounding and death [1–3]. The vast majority of aggressive encounters, however, do not involve fighting. Rather, contenders most often settle confrontations by performing threat displays, which typically lead to one of the participants retreating [4–6].

Early theoretical analyses readily recognized the difficulty with threat displays: it pays all individuals including weak ones to threaten others if threat leads to the challenger withdrawing [7]. The only solution to this theoretical dilemma was to assume that threat either allows fair assessment of fighting ability or indicates willingness to escalate into fighting. In the latter case, contenders must use threat reliably in order to avoid costly loss against stronger opponents. This implies a high positive correlation between threat displays and fighting abilities [8–10].

While the theoretical challenge may have been resolved, the models on aggressive signals and fighting inspired critical reassessment [11] as well as new experiments designed to quantify the information conveyed by aggressive signals. Some studies examined whether threat displays signalled tendencies to escalate into fighting [12,13]. Most notably, in a few species of song birds, soft song was significantly associated with subsequent attack [14–17]. We know, however, of no studies that have examined the genetic correlation between threat and fighting.

A complementary approach for predicting the strength of association between threat signals and fighting is to consider likely mechanistic constraints. One would expect similar genetic, physiological and neuronal mechanisms to mediate aggressive signals and actual fighting. This could lead to a positive genetic correlation between threat and combat. Indeed both phenotypic and genetic correlations among traits are ubiquitous [18–22]. We lack data, however, about the genetic correlation between threat and fighting.

To further our understanding of the association between threat displays and fighting, we used fruit flies (*Drosophila melanogaster*), which are a highly tractable model system for quantifying aggression. When placed in settings that allow resource defence, male fruit flies defend attractive food patches both in

the absence and presence of females. The flies perform well-characterized aggressive behaviours. The sole, prominent aggressive signal is wing threat, in which a fly raises his wings at 45° toward his opponent. Fighting primarily includes lunging, where the aggressor hits his opponent with his forelegs, and occasionally more escalated combat including boxing, where both males rear up on their hind legs and strike each other with their forelegs; and tussling, involving the males stumbling over each other [23–27].

One of the unique tools available in fruit flies is the *Drosophila* Genetic Reference Panel (DGRP), a set of sequenced, fully inbred lines, which has been used widely for investigating the genetic basis of various traits including aggression [28–33]. We thus used a subset of the DGRP lines to test the genetic correlation between threat and fighting. Both signals of aggression and fighting itself may vary as a function of the social context [26,34]. Hence we conducted two experiments, each assessing threat and fighting in one of two realistic social settings. In natural sites with small fruits and low to moderate fly densities, capable males attempt to monopolize fruit through aggression and mate with females that seek food and egg laying sites. Sometimes, males and females co-occur at the fruits, while at other times, males may encounter only other males at the fruits [27]. We simulated these two relevant scenarios by allowing males in one experiment to interact with both males and females but to encounter only males in the other experiment. In both cases, we predicted a positive genetic correlation between threat displays and fighting.

2. Methods

(a) General

We used *Wolbachia*-free lines from the DGRP [33,35] and housed them in standard fly vials with 5 ml of our standard food medium (1 l = 90 g sucrose, 75 g cornmeal, 10 g carrageenan, 32 g yeast and 2 g methyl paraben dissolved in 20 ml ethanol). We maintained the flies in an environmental chamber at 25°C and 50% relative humidity on a 12 h light : dark cycle with the lights turning on at 10.00 h. In order to lessen deleterious inbreeding effects, we created F1 hybrid flies for the experiments by crossing males from 24 distinct DGRP lines to females from a single line (DGRP-83) [36]. To generate the hybrids, we collected virgin females of DGRP-83 within 8 h of eclosion using light CO_2 anaesthesia and housed them in groups of 15 per food vial sprinkled with live yeast to stimulate egg laying. Once females were 3–5 days old, we transferred them in groups of eight to new vials containing food and live yeast and added to each vial five males of 1–4 days old from one of the DGRP lines. We transferred these parental flies of the 24 hybrid crosses into new food vials with live yeast daily, and scraped excess eggs from the vials to ensure a consistent rearing density across lines. Eleven days after egg laying, we collected by aspiration the F1 hybrid flies (hereafter genotypes) within 8 h of eclosion to ensure virginity and aspirated the focal flies individually into food vials.

(b) Aggression experiments

We conducted two experiments to assess threat and fighting under the two realistic settings of males at a food patch that either has or lacks females. We tested the focal flies when they were 4 days old within 2 h of the onset of the light period. This period is associated with the morning peak of fly activity in most studies [27,37]. Our test arenas consisted of polystyrene Petri dishes that were 35 mm in diameter and 8 mm high. To restrict the flies to the floors of the arenas, we coated the walls and ceilings with Surfasil (Thermo Fisher, Ottawa, Ontario, Canada). We covered the floor of each arena with filter paper and placed at its centre a small, circular food patch made of standard medium. In experiment 1 (females present), the food patch was 7.5 mm in diameter and 1.5 mm high and coated with a suspension made of 3 g live yeast and 100 ml grapefruit juice. In experiment 2 (females absent), the food patch was 5 mm in diameter and had at its centre a 3 mm ball of paste made from a mixture of 5 g live yeast and 10 ml grapefruit juice. On each test day, we ran 1–4 sets of test trials for each of the genotypes with the order of genotypes randomized and counterbalanced. Experiment 1 included 16 DGRP genotypes and 443 trials (25–30 replicates per genotype). In experiment 2, we increased the number of DGRP genotypes to 24 and had a total of 361 trials (14–16 replicates per genotype).

In experiment 1 (females present), we aspirated into each arena two focal males and a recently mated female. Within each test arena, we always placed flies of a single genotype that had developed in distinct food vials to avoid familiarity. The female had mated once with a same-genotype male just

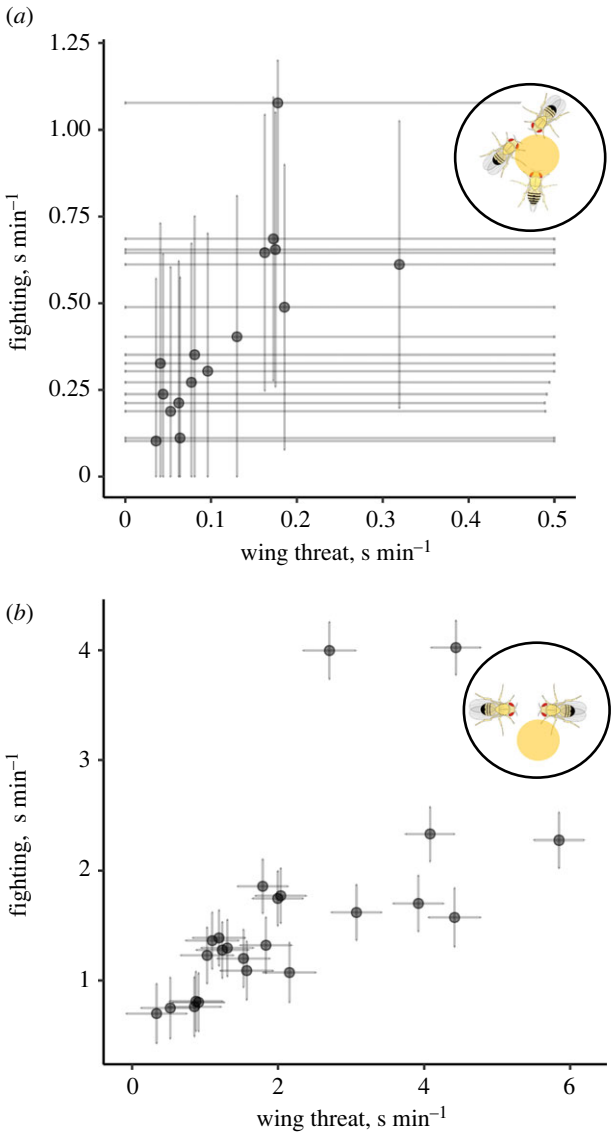

Figure 1. The genetic correlation between wing threat and fighting among distinct genotypes in the presence (*a*) and absence (*b*) of females. Note the different trait value ranges between (*a*) and (*b*). Each point shows conditional genotypic means ± 95% CI. The inserts illustrate the arenas with and without females. Males are identified by the black tip of their abdomen. $N = 443$ trials with 16 genotypes and 361 trials with 24 genotypes, respectively.

Table 1. Trait means (response scale), genetic variances, residual variances and heritabilities (latent variable scale).

experiment	behaviour	*trait mean (95% CI)*	V_G (95% CI)	V_r (95% CI)	H^2
females present	threats	0.093 (0.033,0.26)	1.30 (0.50, 3.22)	3.3 (2.4, 4.4)	0.28
females present	fighting	0.34 (0.12, 0.95)	1.30 (0.52, 3.28)	3.4 (2.7, 4.4)	0.27
females absent	threats	1.64 (0.98, 2.77)	1.24 (0.64, 2.50)	1.4 (1.0, 1.9)	0.47
females absent	fighting	1.4 (0.89, 2.19)	0.56 (0.44, 1.12)	0.64 (0.46, 0.90)	0.46

prior to the test. Recently mated females typically do not remate, and we excluded only two trials owing to mating. Female presence, however, may alter the dynamics of male aggression [26,34,38]. In experiment 2 (females absent), we aspirated into each arena just two focal males of the same genotype. We allowed the flies 5 min to acclimate and then video recorded them for 10 min using webcams (Logitech HD Pro C920). After the experiments, observers blind to fly genotype recorded from the videos the duration of wing threat and fighting [23,26] using BORIS, an event-logging software [39]. We recorded wing threat, which consists of the aggressor raising his wings at 45° toward his opponent. For fighting, we recorded lunging, which is the most common component of physical aggression and characterized by the aggressor making a fast movement and hitting with his head his opponent. We also recorded less frequent elements of fighting including holding, in which the aggressor uses his forelegs to grasp his opponent; boxing, where both males rear up on their hind legs and strike each other with their forelegs; and tussling, involving the males stumbling over each other (see [27], electronic supplementary material video S1 0:51). In the females-present experiment, males spent some time pursuing the females, which, theoretically, could reduce their time available for aggression. We nevertheless reported the total time males devoted to threat and fighting in order to avoid potential biases.

(c) Statistics

We analysed the data with R v. 4.0.2 [40]. We used tidyverse v. 1.3.2 [41] to organize data and generate figures, and constructed generalized linear mixed-effects models (Tweedie distributed with a log link) using glmmTMB v. 1.1.4 [42]. We visually examined the distributions of raw data and used the DHARMa package v. 0.4.5 [43] to aid in model diagnostics. Each model included threat duration and fighting duration as dependent measures, and genotype, day, and trial indentification (ID) as random effects. See the electronic supplementary material for further details.

Broad sense genetic variance ($V_G = 2{*}V_{DGRP}$), genetic correlations (r_G), and heritabilities (H^2) were extracted and computed from estimates directly from glmmTMB. We multiplied the among-DGRP hybrid variance component by 2 to account for the shared maternal line of the DGRP hybrids. We reported broad sense heritabilities because our protocol, in which we crossed males from all lines with females from a single line, did not allow us to exclude non-additive genetic effects. H^2 was estimated as $V_G/(V_G + V_E) = 2\sigma_l^2/(2\sigma_l^2 + \sigma_e^2)$, where V_G is genetic variance, V_E is environmental variance, σ_l^2 is the among-DGRP hybrid variance component, and σ_e^2 is the error variance [44–46]. Trial level variances were used as a proxy for residual variances. Estimating quantitative genetic parameters for complex generalized linear mixed models (with respect to the scale of the latent variable versus the data scale) remains challenging for situations such as semi-continuous, 'zero-inflated' positive-valued data, as observed here. As such we only include these estimates on the latent variable scale, which will have higher estimates of H^2 relative to the original data scale due to the impact of exponentiation back to the response scale [47,48]. Data and code to reproduce analyses are available at: https://doi.org/10.6084/m9.figshare.21565722.v1 [49].

3. Results

In the experiments with (figure 1*a*) and without (figure 1*b*) females, there were relatively high genetic correlations (r_G) between wing threat and fighting ($r_G = 0.80$, 95% CI:[*0.15, 0.93*] and $r_G = 0.74$ [*0.25, 0.89*] respectively). For both data sets, we compared full model fits to their corresponding models constrained with $r_G = 0$. These comparisons are consistent with the unrestricted models being preferred for both experiments (females present: LR = *8.75*, d.f. = *1*, $p = 0.003$; females absent: LR = *14.2*, d.f. = *1*, $p = 0.0002$;). Broad sense genetic variances and heritabilities for both threat and fighting were moderate (table 1).

While males spent much less time threatening than fighting when females were present (threat/fighting ratio = 0.27, with 95% CIs of 0.19–0.39, $t = -7.7$, $p < 0.0001$), they spent similar times on threat and fighting when females were absent (threat/fighting ratio = 1.18, with 95% CIs of 0.89–1.55, $t = 1.2$, $p = 0.23$, figure 1).

4. Discussion

Our experiments indicated relatively high genetic correlations between levels of threat and fighting (figure 1). The fact that threat and fighting are highly positively correlated agrees with theoretical predictions that signallers should modulate their threat in relation to their willingness to escalate into combat. Failure to do so can result in the costly defeat of weak individuals that signal strength [8–10]. Although fruit flies do not possess weapons that can inflict injuries, their fighting is nevertheless costly as it leads to reduced lifespan [50]. The theoretical predictions, however, implicitly assume that threat and fighting may vary independently. In reality, however, there are good reasons to assume that mechanistic constraints maintain at least a moderate positive genetic correlation between threat and fighting. Genetic correlations, even between apparently unrelated behaviours, are prevalent [20,22,51,52]. In the case of aggression, one can readily envision that the same genetic networks, neural networks and endocrine mechanisms modulate both threat and fighting. Finally, while we focused on the durations of threat and fighting, it is possible that other features, such as the tendency to escalate after threat, or actual ability to win a fight following a threat may be more informative.

The values of genetic correlations between threat and fighting in our experiments (0.74 and 0.80) were somewhat higher than the average of about 0.6 reported for other behaviours [20]. While such high genetic correlations may pose a

constraint on the independent evolution of threat and aggression, previous studies indicate that high genetic correlations still allow for relatively rapid evolutionary change in one of the correlated traits [19,53,54]. Intriguingly, a fruit fly study suggested that one of the five serotonin receptors they possess, $5HT_{1a}$, modulates wing threat, while another serotonin receptor, $5HT_2$, modulates fighting [55]. Overall though, we still do not understand how the few neurotransmitters that modulate aggression and their multiple receptors orchestrate threat and fighting [56]. Nevertheless, our results, along with the rapidly increasing mechanistic knowledge about fruit fly aggression, open up exciting avenues for future research. Specifically, given the genetic variation in the threat and fighting, one can artificially select for lineages of flies that either display few aggressive signals but eagerness to fight or vice versa. Then research on the evolved lineages can assess both the genetic and neurobiological changes in such flies and their functional effects on social interactions. All the protocols and tools for such research are currently available [28,57–59].

Males showed a much lower ratio of threat to fighting in the presence than in the absence of females (figure 1). It seems that, despite the high genetic correlation, males can adjust the ratio of threat to fighting in response to relevant features of their social environment. Males spent some time courting females and less time in both threat and aggression in the presence of the recently mated females (panels (*a*) versus (*b*) in figure 1). While we cannot explain the difference in threat to fighting ratio in the females present and females absent experiments, audience effects on behaviour have been documented in a variety of species including fruit flies [26,34,38,60,61].

In sum, we documented a high positive correlation between aggressive signals and actual fighting. This opens up exciting opportunities for further investigations on the mechanistic and functional bases underlying the association between aggressive signals and fighting in a leading model system.

Data accessibility. Data and code to reproduce the analyses are available in the electronic supplementary material [49].

Authors' contributions. C.M.B.: conceptualization, investigation, writing—original draft, writing—review and editing; I.S.: investigation, writing—review and editing; I.D.: software, visualization, writing—original draft, writing—review and editing; R.D.: conceptualization, methodology, project administration, supervision, visualization, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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