Objective and subjective components of resource value in lethal fights between male entomopathogenic nematodes

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Males sometimes engage in fights over contested resources such as access to mates; in this case, fighting behaviour may be adjusted based on the value they place on the females. Resource value (RV) can have two components. First, males can assess the quality of females, which constitutes an objective assessment of RV. Second, internal state such as previous mating experience can also influence motivation to fight thus constituting a subjective assessment of RV. If mating opportunities are scarce and available females have a major impact on the lifetime reproductive success of males, then fighting can be fatal; in this situation it is uncertain whether males would adjust fighting behaviour based on RV. We found that both female quality, that is, virginity (objective component of RV) and male mating status (subjective component of RV) influenced fighting intensity between males of the entomopathogenic nematode *Steinernema longicaudum* which engage in lethal fights. Male nematodes were more likely to engage in fighting and fought longer and more frequently in the presence of virgin (high-quality) females than in the presence of mated (lower-quality) females. Male mating status was also found to influence fighting behaviour: mated males were the winners in staged fights between mated and virgin males. Mated males may have superior fighting ability (greater resource-holding potential), but RV asymmetries between mated and virgin males cannot be excluded. Males were more likely to win when they were resident, but we did not find a significant interaction effect between male mating and residency status.
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Animals frequently engage in contests over resources that impact their fitness, such as mates, territories or nesting sites (Andersson, 1994). Among the most important factors influencing the extent and outcome of contest behaviour are the fighting ability (resource-holding potential, RHP) of the contestants, the value of the resource (RV) and the costs of fighting (Parker, 1974; Arnott & Elwood, 2008; bib_Hardy_and_Brifa_2013·Hardy & Brifa, 2013). The RHP of the contestants may depend on their weaponry, energy reserves and body size (Parker, 1974; Arnott & Elwood, 2009; Hardy & Brifa, 2013; Rico-Guevara & Hurme, 2019). The RV has two components: objective and subjective. The objective components are the intrinsic properties of the resource that will yield a certain fitness gain to a successful competitor, while the subjective component is the value of the resource for each contestant depending on its own internal state and prior experience (Enquist & Leimar, 1987; Arnott & Elwood, 2008). These components of RV may simultaneously influence the ultimate value of the resource (Stockermans & Hardy, 2013).

Objective and subjective components of RV have been widely studied in contests between males for mates, where the contested resource is receptive females. When females are scarce, males may be more motivated to fight, but this motivation can also depend on female quality which can be based on size (larger females are more fecund) and nearness to moult or mating status (Crespi, 1988; Ancona, Drummond, & Zaldivar-Rae, 2010; Arnott & Elwood, 2008; Dick & Elwood, 1990; Hoefler, Guhanarayan, Persons, & Rypstra, 2009; Kasumovic, Mason, Andrade, & Elias, 2011; Keil & Watson, 2010). Furthermore, males' internal state, such as their own mating status, can influence subjectively the value that they place on the contested resource (i.e. mates; Arnott & Elwood, 2008). Mated males may become less aggressive and more likely to lose at contests due to the energetic cost of mating (Brown, Chimenti, & Siebert, 2007, 2006; Judge, Ting, Schneider, & Fitzpatrick, 2010). On the other hand, previous mating experience has been shown to increase a male's aggression and fighting

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success in several animal taxa because of changes in the male's self-assessment of RHP and perception of RV that are similar to winner/loser effects (Dugatkin; Dugatkin 2011; Killian & Allen, 2008; Yasuda, Matsuo, & Wada, 2015; Zhang, Ai, & Li, 2019). If an animal wins a contest then its perception of fighting ability increases and it is more likely to initiate future fights and win them, whereas losers lower their RHP perception, are less aggressive and more likely to lose (Hsu, Earley, & Wolf, 2006). Mating status is also related to the residual reproductive value (number of future offspring) which is predicted to influence male investment in fighting (Kemp, 2006). An unmated male can be more aggressive and more motivated to fight because future mating opportunities are of greater value to him, whereas a mated male may be less willing to engage in costly fighting (Kemp, 2006).

Fighting is costly, risking injury and using time and energy that could be used in mating or other activities (Maynard Smith & Price, 1973; Briffa & Sneddon, 2007). As the value of the contested resource increases, the cost of fighting can also increase (Enquist & Leimar, 1987; Kokko, 2013). If the contested resource has a major impact on the lifetime reproductive success of the contestants, such as limited females, then fights can escalate to serious injury and even death (Enquist & Leimar, 1987, 1990; Maynard Smith & Parker, 1976). Lethal male fighting has been recorded in some vertebrates (e.g. Piper, Walcott, Mager, & Spilker, 2008) and numerous arthropods, particularly arachnids (DeCarvalho, Watson, & Field, 2004; Sato, Egas, Sabelis, & Mochizuki, 2013) and hymenopterans (Anderson, Cremer, & Heinze, 2003; Matthews, Gonzalez, Matthews, & Deyrup, 2009; Cook & Bean, 2006; Hamilton, 1979; Innocent et al. 2007, 2011) and lately in entomopathogenic nematodes (Zenner, O'Callaghan, & Griffin, 2014; Kapranas, Maher, & Griffin, 2016). Whereas contested resource availability, such as potential mates, is critical in prompting lethal fights (Enquist & Leimar, 1990), it is unclear to what extent the contestants also assess the quality of resources in such species. When mating opportunities for males are extremely limited then their motivation to fight would be expected to be independent of resource quality even if the odds of dying in a fight are high (Enquist & Leimar, 1990). Among parasitoid hymenopterans with lethal male combat, some species adjust fighting behaviour according to the value of the resource (Liu & Hao, 2019), while others do not (Innocent et al., 2011).

Here we investigated the effect of objective and subjective RV on lethal contests of male entomopathogenic nematodes, *Steinernema longicaudum*. We manipulated the mating status of the contestant males and the quality of the contested resource by staging dyadic contests in a controlled environment (drops of haemolymph of their insect host). First, we assessed how the presence/absence and quality of available females (objective components of RV) influenced fighting behaviour between mated males. We predicted that the presence of a female, especially a virgin, would increase the frequency and/or severity of fighting in *S. longicaudum*. Then, we assessed whether male mating status (subjective components of RV) influenced the contest outcome. We did not have a directional prediction of how mating would influence fighting, but we hypothesized that either mated or virgin males would have a consistent advantage over the other, and this would provide insights regarding how male nematodes assess the value of their mates and whether mating status influences fighting ability.

METHODS

Nematode Reproductive Biology and Lethal Fighting

Infection by entomopathogenic nematodes including *S. longicaudum* begins when free-foraging infective juveniles enter an insect host and release their symbiotic bacteria which turn the host insect into a carcass. In *S. longicaudum*, infective juveniles develop into amphimictic males and females, with a sex ratio that is slightly female biased (Alsaiyah, Ebssa, Zenner, O'Callaghan, & Griffin, 2009). Two or more generations develop until the carcass is crowded, prompting the production of infective juveniles which exit in search of other hosts (Kaya & Gaugler, 1993; Dillman & Sternberg, 2012). In *S. longicaudum* males are aggressive towards each other and engage in lethal fights (Zenner et al., 2014). During male–male encounters, an aggressor coils its tail end around a victim, with the copulatory spicules towards the victim's body. If not gripped close to the tail, the victim may counterattack, wrapping its tail around the aggressor. Grappling encounters frequently end when the grasped male ceases to move part or all of its body, which may happen within minutes (Zenner et al., 2014). Such worms usually die, although partially paralysed worms occasionally recover movement. The cuticle is sometimes punctured, but paralysis and death also occur without puncture, apparently due to damage to internal organs.

Nematode Cultures and Behavioural Assays

Steinernema longicaudum (strain CB2B) cultures were routinely maintained using standard procedures by passage through late-instar *Galleria mellonella* (wax moth) larvae (Kaya & Stock, 1997) at 27 °C. Infective juveniles were stored in tap water at 20 °C. We obtained adult males for our experiments by placing infective juveniles individually in 25 μl hanging drops of haemolymph from *G. mellonella* larvae which provides a suitable environment for development to adulthood (Kaya & Stock, 1997; Zenner et al., 2014). The hanging drops were placed on the lid of a Petri dish which was then inverted over a water-filled Petri dish (6 cm diameter) to prevent desiccation. The infective juveniles in the hanging drops develop to adulthood within 3 days at 27 °C. Adult males can be distinguished by their copulatory spicules and their smaller size whereas females are larger and bear vulva. We used 3-day-old males and females in our experiments.

Experiment 1: Female Presence and Quality

All males used in this experiment were mated. We placed each individual naïve male for 16–20 h with two naïve females to mate. We then removed the females, and successful mating was subsequently confirmed by the production of progeny. We removed two adult males from their hanging drops and placed them immediately, in pairs, back in the drop from which one member of the pair had been taken. Thus, one worm was 'resident' and one an 'intruder', but the identity of each was not tracked, as there were no clear visual differences between them. Worms were assigned to the following treatments: (1) two mated males with a mated female ($N = 34$); (2) two mated males with a virgin female (same age as the mated female; $N = 31$); (3) two mated males without a female ($N = 35$). A mating attempt was recorded when a male coiled around the female at the vulva; insertion of spicules into the vulva was difficult to observe due to this coiling. A fight was recorded once one male coiled tightly around another and then let go. Continuous observations were made for 30 min during which the latency to the first fight, the incidence of fighting (whether at least one fight occurred), number and (for a subsample) duration of fights were recorded. Paralysis and/or death of the males was recorded at the end of the observation and after 24 h.

Experiment 2: Male Mating Status

In *S. longicaudum*, males that have been with a female can be distinguished from those that have not by the presence of sperm in the seminal vesicle, visible through the body wall (Ebssa, Dix, &

Griffin, 2008); thus, mating status of individual males in a pair could be recognized. In this experiment, we placed together an adult male that had mated and a virgin one. Mated males were obtained by placing two females in the drop of an individually reared adult male for 24 h, after which the females were removed to a different drop and observed for later progeny production. We set up pairs of one mated and one virgin adult male either in the drop in which the mated male had been reared and mated ($N = 21$) or in the drop in which the unmated male had been reared ($N = 24$). In this case, since mated and unmated males differed in appearance, the identity of 'resident' and 'intruder' could also be traced. After 24 h we observed which worms showed signs of paralysis or death. Individual males were identified as the mated or virgin member of the pair by examining for the presence of sperm using a Nikon Optiphot microscope (x40).

Statistics

To analyse factors influencing the number of fights as well as copulations observed in our assays and the probability of paralysis/ death we used generalized linear models as described in Briffa et al. (2013). To test the hypothesis that objective RV affects fighting behaviour, we used a log-linear analysis which is appropriate for small count data, to assess how female presence and mating status (predictor variables) affected the number of fights (dependent variable). Post hoc tests between different treatments whenever applicable were adjusted with the Bonferroni correction. The probability of paralysis and/or death at the end of the 30 min observation period and 24 h after observations was explored with logistic analysis. Duration of fighting in different treatments (no females, mated and virgin females) was analysed with a nonparametric Kruskal—Wallis test and latency to fighting was analysed using survival regression analysis (Moya-Larano & Wise, 2000). In the second experiment, we compared the incidence of paralysis/ death in contests staged in drops where the resident male was mated versus drops where the resident was a virgin male by using a Fisher's exact test.

For those pairs in which paralysis or death occurred, we used a logistic analysis by randomly picking a focal male (either mated or virgin) in each replicate and tested the effect of residency and mating status (as factors) and their interaction on the probability of the focal male winning the contest (Briffa et al., 2013). All analysis was performed in SPSS v.21 (IBM, Armonk, NY, U.S.A.).
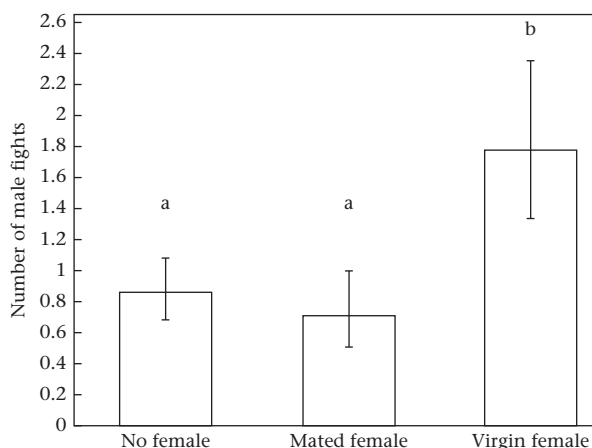
Ethical Note

The experiments were conducted in accordance with Science Foundation Ireland's policy concerning use of animals in research (outlined in Directive 2010/63/EU) implemented by the Health Products Regulatory Authority (HPRA), the competent authority in Ireland responsible for the protection of animals used for scientific purposes. All experimentation reported in this work abides to the principles of replacement, reduction and refinement as endorsed by the ASAB/ABS Guidelines for the Use of Animals in Research.

RESULTS

Experiment 1: Female Presence and Quality

Significantly more fights were observed in the presence of virgin females than in the presence of mated females or in the absence of females ($F_{2,97} = 5.79$, $P = 0.004$; Fig. 1). This was partly explained by the higher incidence of fighting in the presence of a virgin female (74.2%) than with a mated or no female (44.1 and 42.9%, respectively). In addition, the latency to fight was shorter in the presence of virgin females than in the presence of mated females or in the absence of females (Cox's proportional hazards analysis: risk = 0.448, 95% confidence interval = 0.233 − 0.860, $P = 0.016$; Fig. 2), and fights also tended to last longer when there was a virgin female present (Kruskal—Wallis test: $H_2 = 5.56$, $P = 0.062$; Fig. 3).

The presence or quality of a female did not influence the incidence of paralysis at the end of the 30 min observation period ($G_2 = 1.437$, $P = 0.487$) when on average 6% of pairs had one male paralysed; nor did it affect the incidence of paralysis and/or death after 24 h ($G_2 = 2.839$, $P = 0.242$), where overall 97% of pairs had at least one male either paralysed or dead (with dead males in 90.9% of pairs). Single males routinely suffer 3% mortality within 24 h (Zenner et al., 2014).

Moreover, the number of copulations (mating events at vulva) was higher when the female was virgin than mated (mated females: mean ± SE = 0.88 ± 0.18; virgin females: mean ± SE = 1.74 ± 0.26; $F_{1, 63} = 7.322$, $P = 0.009$).

Experiment 2: Male Mating Status

After 24 h, paralysis or death was recorded in all 21 drops where the resident male was mated, but in just 70.8% (17/24) of the drops where the virgin male was resident (Fisher's exact test: $P = 0.01$). For the 38 pairs where there was paralysis or death after 24 h, we investigated whether male mating status and prior residency
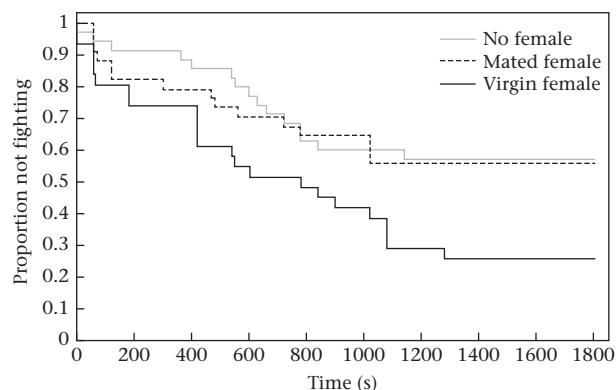

Figure 1. Fights between two mated males in the presence or absence of a female within 30 min. Bars show average values with asymmetrical, Poisson-distributed errors. Bars with the same letter are not significantly different (post hoc multiple comparison tests with Bonferroni adjustment, $\alpha = 0.05$).

Figure 2. Latency to first fight between two mated males in the presence or absence of a female within 30 min.
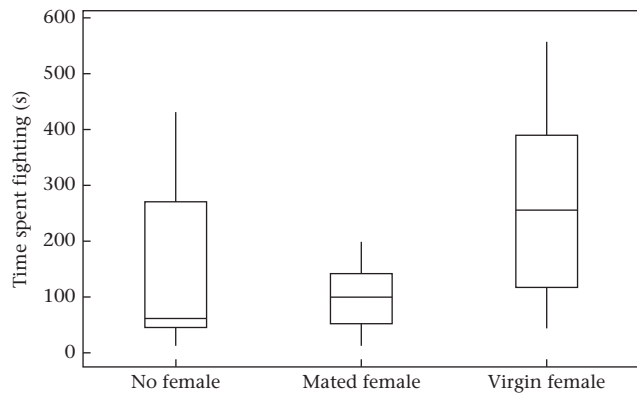
Figure 3. Time spent fighting by two males in the presence or absence of a female within 30 min. Box plots indicate the median (horizontal line), 25th−75th percentiles (boxes) and ranges for the bottom and top 25% of the data values (whiskers).

Figure 4. Probability of winning for mated and virgin males in pairs of one mated and one virgin adult male set up either in the drop in which the mated male had been reared and mated or in the drop in which the unmated male had been reared. Bars show estimated average values with asymmetrical, binomially distributed standard errors.

affected the contest outcome. Mated and resident males were more likely to win (logistic regressions for male mating status: $G_{1,34} = 75.13$, $P < 0.001$; male residency: $G_{1,34} = 10.313$, $P < 0.001$; Fig. 4). The effect of male mating status was particularly strong: mated males won 92.1% of fights overall. The interaction between male mating status and drop residency was not significant ($G_{1,34} = 0$, $P = 0.991$).

DISCUSSION

The presence of a female can lead to escalated male fighting, as shown in spiders and parasitoid wasps (Wells, 1988; Jackson, Walker, Pollard, & Cross, 2006; Liu, Wei, Tian, & Hao, 2017). Moreover, the motivation to fight and the intensity of fights, and consequently the cost of fighting, are adjusted according to the perceived value of the resource (Parker, 1974; Maynard Smith & Parker, 1976; Arnott & Elwood, 2008; Enquist & Leimar, 1987). In our study we found that not only the presence but also the quality of females had an influence on fighting behaviour of male *S. longicaudum* nematodes: fighting was more intense in the presence of a virgin female, during our 30 min observation period. In other empirical studies, males similarly exhibited significantly more aggressive behaviours, and fights were more intense in the presence of virgin females than mated ones in parasitoid wasps, *Anastatus disparis* (Liu & Hao, 2019), wolf spiders, *Pardosa milvina* (Hoefler et al., 2009) and Sierra dome spiders, *Neriene litigiosa* (Keil & Watson, 2010). The fact that a virgin (but not a mated) female increases competition between male *S. longicaudum* suggests either that females mate only once or that there is sperm competition with first-male precedence (Birkhead & Møller, 1998; Simmons, 2001). Sperm precedence has been documented in nematodes including *Caenorhabditis elegans* (LaMunyon & Ward, 1995; Ward & Carrel, 1979). Female steinernematids no longer attract males over a distance when mated (Lewis, Barbarosa, & Gaugler, 2002; Hartley, 2017), and in our experiment mated females received fewer mating attempts (sperm transfer was not confirmed) than virgins, suggesting at least a reduction in attractiveness. They have at most a short window of availability for mating, with a period of egg laying succeeded by egg hatch in utero and subsequent death ('endotokia matricida'; Poinar, 1990). The exceptionally large macrosperm of *S. longicaudum* are suggestive of intense sperm competition (LaMunyon & Ward, 1999). Because the cost of fighting is high, selection favours individuals that can assess the value of the resource and adjust their competitive behaviour

accordingly (Arnott & Elwood, 2008; Maynard Smith & Parker, 1976; Parker, 1974). When mates are limited over time and space and their current value is equal to or higher than that in the future, then a major part of a male contestant's lifetime reproductive success is at stake and it is predicted that fights can escalate to death (Enquist & Leimar, 1987, 1990). In such cases, fights might ensue irrespective of the quality of the mates, since future opportunities for reproduction are low, but our results show that male *S. longicaudum* nematodes assess the quality of the females and adjust their fighting behaviour. We suggest that mated females, owing to either one-off mating or intense sperm competition, might simply not represent a valuable resource worth fighting to death for.

Differences in the quantity and quality of food or mates lead to an 'objective' assessment of the value of the contested resource, whereas internal state dictates its subjective assessment (Enquist & Leimar, 1987; Arnott & Elwood, 2008). In dyadic fights between mated and virgin males in the present study, mated males were more competitive, resulting in paralysis and death of the virgin male in over 90% of cases. Mating could increase fighting skills in nematodes, since similar coiling movements of males around females during copulation are used to lock, paralyse and consequently kill their opponents. Increased fighting skills increase RHP (Briffa & Lane, 2017). Alternatively, mating in males can result in an increased assessment of fighting ability or an overestimation of the contested RV (Killian & Allen, 2008; Yasuda et al., 2015). Mating experience has similar effects to 'winning a fight' experience, which is expected in turn to raise the subjective value of a female in future contests (Hsu et al., 2006).

Contests are frequently asymmetrical; one such asymmetry is between territory owners or residents and intruders. In such cases, it is usually the resident that wins (Hardy & Brifa, 2013). Owner−intruder asymmetry is less likely to affect fighting outcome when the ratio of future to current reproduction is low (Enquist & Leimar, 1990). The relationship between male residency and increased competitive ability that was observed in our experimental conditions might be explained by the fact that the resident males were more adapted to the environment (which included bacterial/female pheromone odours) of the drop in which they reached adulthood than the intruder males. The extent to which residency effects for male entomopathogenic nematodes occurs in nature is unclear as they compete in a restricted space, an insect

carcass, in which it might sometimes be difficult to claim residency. However, larger insect carcasses such as those of *G. mellonella* may be large enough to allow some degree of localization of residents, because at the time the invading nematodes are adult, the insect carcass retains internal structural integrity, allowing physical compartmentalization of individuals within it. Regions of the carcass could also be chemically differentiated, especially if colonized by nematodes derived from different lines of free-foraging infective juveniles, for example those that exited from different natal host species or have different bacterial strains and/or chemical signatures. Another source of odours that could differentiate space within a carcass is the female residents. Female *S. longicaudum* are relatively immobile, tending to coil in situ, while males are active and attracted by female pheromones (Hartley, 2017). Thus, it is likely that a 'territory' or patch is defined by the area of the carcass influenced by a female's odour, at least in a carcass with a relatively low population density, and such a patch could be defended by a resident male. The increased probability in experiment 2 of the resident male winning a fight in drops in which the resident was mated might be due to residual pheromones from virgin females increasing the perceived RV of the drop. Mated *S. longicaudum* males fight more in the presence of a virgin female, as shown in experiment 1, and female *S. longicaudum* secrete pheromones that affect males behaviourally and physiologically even when no female is present (Ebssa et al., 2008; Hartley, 2017). Female pheromones increase aggression in male arthropods including crabs, (Smith, Huntingford, Atkinson, & Taylor, 1994; Sneddon, Huntingford, Taylor, & Clare, 2003), crickets (Buena & Walker, 2008) and parasitoids (Liu & Hao, 2019). However, female pheromones cannot be the explanation for the increased probability of the resident winning in the drops where it was virgin, since these drops had not previously held a female. Both mated and virgin males were more likely to win when resident, but since mated residents may have experienced pheromone, but virgin residents did not, the lack of an interaction between the effects of male mating status and residency may in fact point to an asymmetrical effect of female pheromone on the aggression of mated and virgin males. We have also observed female presence having a differential effect on fighting intensity of unmated and mated *S. longicaudum* males (Kapranas & Griffin n.d.). These differential effects of female presence or pheromones may be related to the fact that *S. longicaudum* that have not been exposed to females are not immediately ready to mate (Ebssa et al., 2008).

Fatal fighting is expected to ensue whenever the value of the resource outbalances the risk of injury, as is the case for entomopathogenic nematode males that have to fight for access to females that are limited in space and time. Our study shows that competitor males adjusted their fighting behaviour according to the objective (presence and quality of females) and subjective (male mating experience) components of RV. Male mating experience could increase the male's fighting ability (RHP) but its influence on fighting ability is also confounded by components of RV.

Acknowledgments

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