

Higher relatedness mitigates mortality in a nematode with lethal male fighting

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Abstract

According to kin selection theory, individuals show less aggression towards their relatives. Limited dispersal promotes interactions among relatives but also increases competition among them. The evolution of cooperation in viscous populations has been subject of mainly theoretical exploration. We investigated the influence of relatedness on aggression in males of entomopathogenic nematode *Steinernema longicaudum* that engage in lethal fighting. In a series of *in vitro* experiments, we found that both competitor male group size and relatedness influence male mortality rates. Higher relatedness led to progressively lower rates of male mortality. In experimentally infected insects, wherein large numbers of males and females interact, the proportion of dead and paralysed (= terminally injured) males was higher when infection was established by infective juveniles originating from a mixture of three lines than in those infected by a single line. The results collectively show that *Steinernema longicaudum* males recognize their kin and consequently male mortality rates are lower in groups consisting of more related males. Furthermore, this monotonic negative relationship between aggression and relatedness suggests that kin selection benefits are still substantial even under extreme competition. Our experiments also suggest that kin recognition in entomopathogenic nematodes has a genetic basis rather than being strictly based on environmental cues. We discuss our findings within the theoretical context of the evolution of altruistic/cooperative behaviour in structured populations.

Introduction

Hamilton's theory suggests that altruistically cooperative behaviour is facilitated by high genetic relatedness of altruists to their beneficiaries (Hamilton, 1963, 1964). In broad sense, cooperation (either encompassing altruism or not) can be expected either when individuals interact with their relatives (kin recognition) or when relatedness in local populations is increased because of limited dispersal, a characteristic of viscous populations (Hamilton, 1964). The evolution of social behaviours in viscous populations has gained much theoretical attention; contrary to Hamilton's earlier expectations that altruistic behaviour would evolve in

viscous populations because of high relatedness, later theoretical studies have shown that when competition among relatives is localized then the effect of competition cancels the effect of relatedness (Queller, 1992, 1994; Taylor, 1992a,b; Wilson *et al.*, 1992; West *et al.*, 2002). Empirical work on local competition between relatives, using fig wasps with males that engage in costly fights for access to mates, did not find any relationship between relatedness and aggression (West *et al.*, 2001). A wealth of theoretical studies since tried to find solutions in decoupling competition and relatedness such that helping behaviour could evolve in viscous populations, by considering life history and demographic factors (summarized by Lehmann & Rousset, 2010). However, these studies do not take into account kin recognition mechanisms. A theoretical study inspired by fig wasp biology (Reinhold, 2003) showed that when there is no difference in male fighting ability and when males recognize their kin,

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male–male fighting is expected to peak at intermediate values of relatedness (a dome-shaped pattern), a result that can also be expected via indirect altruistic behaviour, that is spite (Gardner & West, 2004). This prediction has been supported by only two empirical studies on social mites with male–male lethal fights and with bacteria producing bacteriocins (Gardner *et al.*, 2004; Sato *et al.*, 2013), whereas another study in *Melittobia* wasps did not detect such a dome-shaped relationship between relatedness and aggression (Innocent *et al.*, 2011).

In addition, kin recognition mechanisms have been examined thoroughly. Kin recognition can be mediated through prior association, that is individuals become familiar during sensitive times in their ontogeny or have a genetic basis either by phenotype matching, armpit effects or through recognition alleles such as green-beard genes (Penn & Frommen, 2010). In particular, there has been extensive debate over the stability of genetic kin recognition mechanisms as altruistic behaviour tends to eliminate the genetic polymorphism needed in order to operate (Gardner & West, 2007; Rousset & Roze, 2007). Whereas theoretical work has focused on population structure phenomena, empirical work has been mostly focused on kin recognition mechanisms, with little overlap between both processes with few exceptions (Cornwallis *et al.*, 2009).

Parasites competing within patchily distributed hosts are ideally suited to test all these alternative theoretical predictions, that is kin recognition and the directionality of relatedness effects on competition, but so far only haplo-diploid arthropods have been used in empirical studies (West *et al.*, 2001; Innocent *et al.*, 2011; Sato *et al.*, 2013). In this study, we consider entomopathogenic nematodes *Steinernema* spp. which infect their insect hosts as actively foraging infective juveniles (IJs); upon their entrance into their hosts, they release their symbiotic bacteria *Xenorhabdus* spp., which kill and digest the host turning it into a cadaver, and further develop to adult males and females (Kaya & Gaugler, 1993; Dillman & Sternberg, 2012). Adults of both sexes reach sexual maturity at about the same time and mate within the cadaver (Kaya & Gaugler, 1993); their juvenile offspring develop to adults or, under crowded conditions, they develop into IJs which exit the host and search for a new one to colonize. A single cadaver can sustain multiple generations (Kaya & Gaugler, 1993). Males of the entomopathogenic nematode *Steinernema longicaudum* that develop directly from the IJ stage are far more aggressive than males of subsequent generations and engage in escalated fights; they coil around each other, causing excessive injuries such as ruptured cuticle and/or ruptured intestine and constriction of internal organs that lead to paralysis and then high mortality rates (Zenner *et al.*, 2014). Lethal male fighting has evolved in response to the limited mating opportunities and for securing valuable

resources, that is a host, for sustaining the development of their offspring (Enquist & Leimar, 1990; Zenner *et al.*, 2014). A host can be infected either by a group of IJs originating from a single host, single IJs originating from different hosts and all the possible scenarios in between these two extremes, leading to a considerable variation of relatedness among competitor males.

Given the scarcity of empirical systems to test the ongoing theoretical expectations of the opposing influences of competition and relatedness on a local scale, this study system provides a rare opportunity to test alternative hypotheses. In the absence of kin recognition and at extremely local competition, no effect of relatedness on aggression is anticipated. However, if male worms can recognize their kin, then the effect of relatedness on aggression will be either dome-shaped or monotonic, depending on the scale of competition. In this study, we assess whether *S. longicaudum* males recognize kin and we explore how relatedness influences mortality rates (an index of aggression); this is achieved by varying the proportions of related and unrelated males both in male only groups (*in vitro* experiments) and in mixed (male and female) groups (natural nematode culture experiments). By varying the absolute numbers of males present, we also test the effect of competitor number on mortality.

Materials and methods

Steinernema longicaudum CB2B strain was cultured on *Galleria mellonella* larvae at 27 °C with standard procedures (Kaya & Stock, 1997). For our experiments, we used 9 different lines of *S. longicaudum* that were started from a single pair of adults and bred independently for ca. 10 generations (see Appendix S1 for more information). IJs of *S. longicaudum* were stored in tap water at 20 °C. Adult males were obtained by placing IJs individually in 15- μ L hanging drops of haemolymph from *G. mellonella* larvae which provides a suitable environment for recovery from the arrested state of the IJ and further 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 diam.) to prevent desiccation. Typically, it takes ca. 3 days at 27 °C for the IJs to develop to adult males and females, and thus, all males used in experiments 1 and 2 were 3 days old.

Experiment 1

The effect of relatedness on nematode mortality was assessed by placing groups of 6 males of varying relatedness in 25- μ L fresh haemolymph drops. We used four different treatments: (i) groups consisting of males of the same line ('related'), (ii) groups consisting of males from two different lines (three of each line – 'high

relatedness'), (iii) groups consisting of males from three different lines (two of each line – 'low relatedness') and (iv) groups consisting of males from six different lines ('unrelated'). Each line contributed equally within and across different treatments. Overall, there were 36 replicates for each treatment. Mortality was assessed by inspecting visually each drop at 24 and at 48 h.

Experiment 2

In this experiment, we assessed simultaneously the effect of group size and relatedness on male mortality. We formed six different competitor male groups: (a) 2 males from the same line; (b) 2 males each from a different line; (c) 4 males of the same line; (d) 4 males, each from a different line; (e) 8 males from the same line; and (f) 8 males, 2 each from 4 different lines. This cross-factorial design allowed the comparison of mortality rates when males were related to their competitors (single line; treatments a, c and e), were relatively more related to some of their competitors (treatments f) and when they were unrelated to their competitors (treatments b and d). Each line contributed equally within different treatments. Overall, there were 18 replicates for each treatment. Mortality was assessed by inspecting visually each drop at 24 h and at 48 h. The number of alive, dead (curved posture) and paralysed males (males with kinked posture, moving only slightly, being critically injured) was recorded. In addition, 70 single males were placed in fresh hanging drops as controls.

Experiment 3

We examined the effect of relatedness on male mortality in more natural settings by assessing male mortality in crowded conditions (number of potential competitors) and also by taking into account the number of females (mating opportunities) when they infect insects. For this purpose, *G. mellonella* larvae were infected by exposing them individually on filter paper to 100 μL of *S. longicaudum* IJs suspension (approximately 1000 IJs mL^{-1}). Mixed nematode suspensions were made up with equal numbers of three individual lines. The experiment was set up in an orthogonal design in which each line was equally represented both within single line infections and mixed line infections and also across the two treatments (single vs. mixed line infection). There were 27 replicates of each treatment (single line and mixed lines infection). After 24 hrs, the host larvae were removed from the filter paper and placed individually on moist tissue paper in 3.5 cm Petri dishes and stored for a further 24 hrs at 27 °C. The cadavers were then stored at 20 °C and dissected over a 2-day period in full strength Ringer's solution. This time allows for maturity of first generation adults within the host. The number of alive, dead

and paralysed male nematodes (males that moved only slightly, being critically injured) and the number of female nematodes were recorded. We did not differentiate between live and dead females, as dead females were rarely found, in line with previous findings, both *in vivo* and *in vitro*, that female mortality is about 6% and is not affected by number of conspecific males present (O'Callaghan *et al.*, 2014; Zenner *et al.*, 2014).

Statistical analysis

Analysis of factors influencing male mortality and injury in hanging drops and in *Galleria* cadavers were performed with generalized linear models. We used logistic analyses (binomially distributed errors) for proportional mortality data using full models, using where possible empirically estimated scale parameters to account for potential overdispersion and backwards model simplification to arrive on the minimum adequate model (Crawley, 1993; Briffa *et al.*, 2013). *Post hoc* analysis, in experiment 1, was achieved by aggregating factor levels. Mortality rates at 24 h and 48 h in experiments 1 and 2 were analysed separately as data were not independent. Some replicates consisted of exactly the same composition of lines that could generate pseudo-replication driven type I errors (false significance). Therefore, we ran generalized linear mixed models to reconfirm the result of the initial analysis (Appendix S1), but here we report the result of the original analysis because no differences were noted. Possible interactions of several factors (number of males, group composition and number of females) are not included as in every case they were insignificant.

Results

Experiment 1

Mortality rates differed among groups consisting of relatives and nonrelatives in varied proportions (24 h: $F_{3,143} = 7.39$, $P < 0.001$; 48 h: $F_{3,143} = 6.10$, $P < 0.001$; Fig. 1), being higher as groups of males consisted progressively of more unrelated individuals.

Experiment 2

Male mortality rates increased significantly with group size (24 h: $F_{1,107} = 27.89$, $P < 0.001$; 48 h: $F_{1,107} = 28.27$, $P < 0.001$) and also varied with relatedness (24 h: $F_{1,107} = 10.67$, $P < 0.001$, 48 h: $F_{1,107} = 10.29$, $P = 0.002$) being higher in groups consisting of mixed related and nonrelated males than those consisting of only related males (Fig. 2).

An analysis of combined subsets of data of experiments 1 and 2 corresponding to groups of related males only determined that the number of males had a significant positive effect on proportion dead (24 h:

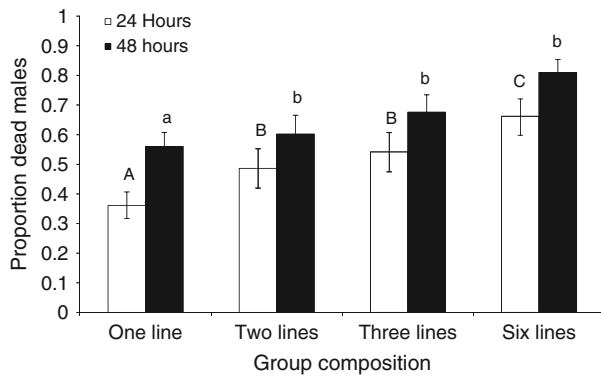


Fig. 1 The influence of group composition on male *Steinernema longicaudum* mortality. Percentages are shown with asymmetrical error bars, and *post hoc* tests within each sampling period (24 h, capital letters; 48 h; lower case letters) were performed by aggregating factor levels.

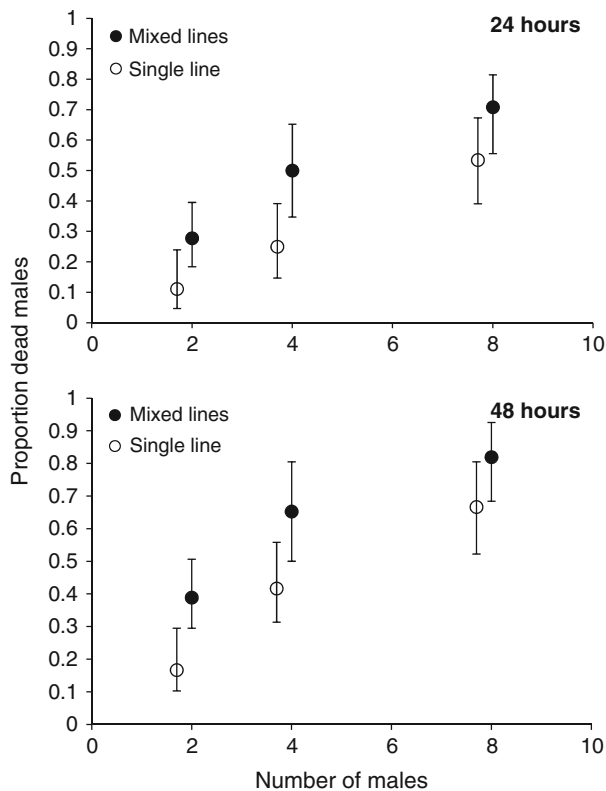


Fig. 2 The influence of relatedness and group size on male *Steinernema longicaudum* mortality. Values are means with asymmetrical errors and within each group size are slightly displaced along the x-axis for clarity.

$F_{1,89} = 15.85$, $P < 0.001$; 48 h: $F_{1,89} = 14.87$, $P < 0.001$, whereas the mortality of males did not differ among different lines (24 h: $F_{8,89} = 1.170$, $P = 0.114$; 48 h: $F_{8,89} = 1.86$, $P = 0.08$) showing that males from different lines show the same level of aggression. More-

over, base mortality rates of males placed singly in fresh hanging drops were 1.4% at 24 h and 10% at 48 h (mortality on multiple male groups on hanging drops was on average 39.8.1% at 24 h and 51.8% at 48 h, Figs 1 and 2).

Experiment 3

The total number of IJs entering cadavers estimated by adding all adult males and females recorded did not differ between single and mixed line infections (single infections: 94.70 ± 8.649 , mixed infections 90.26 ± 8.853 , two tailed $t_{52} = -0.36$, $P = 0.721$). Similarly, the sex ratio (percentage males) was not different between single and mixed line infections (single infection: sr = 44.9%; mixed line infection: sr = 44.9%; $F_{1,53} = 0.45$, $P = 0.504$).

The total number of males had a negative effect on male mortality and injury rates (proportion 'dead' males: $F_{1,53} = 32.92$, $P < 0.005$; proportion 'dead + paralysed' males: $F_{1,53} = 33.11$, $P < 0.001$), whereas the effect of the number of females was insignificant (proportion 'dead' males: $F_{1,53} = 0.04$, $P = 0.847$; proportion 'dead + paralysed' males: $F_{1,53} = 0.02$, $P = 0.876$). The proportion of dead males was unaffected by type of infection ($F_{1,53} = 2.78$, $P = 0.102$); however, the proportion of the 'dead + paralysed' males was significantly affected by type of infection, being higher in the mixed lines infection than in the single line infection ($F_{1,53} = 4.48$, $P = 0.039$, Fig. 3).

Discussion

In the entomopathogenic nematode, *S. longicaudum* males engage in lethal fights for access to females with

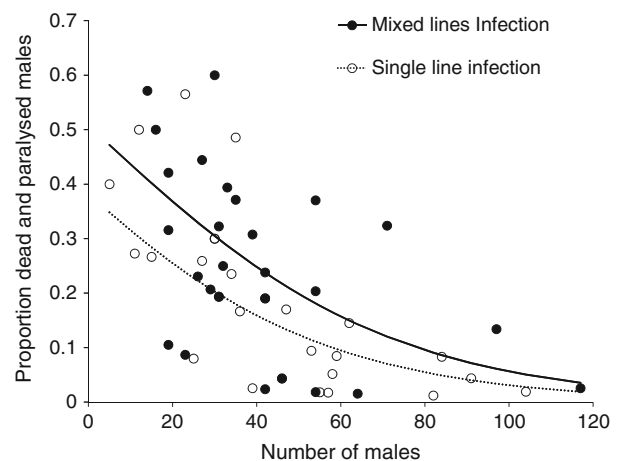


Fig. 3 The influence of male competitors and relatedness in mortality and paralysis rates of competing *Steinernema longicaudum* males.

are limited in space and time (Zenner *et al.*, 2014). Mortality of adult male nematodes when in groups is a direct consequence of their aggressive behaviour towards their competitors (Zenner *et al.*, 2014). Here we show that interactions among more related males lead to lower mortality (and injury) rates consistent with Hamilton's theory (Hamilton, 1963, 1964). Specifically, increased relatedness among fighting males leads to lower mortality rates in groups of only males *in vitro*. In the more natural situation of the insect cadaver and particularly in cases of high competitor density, the effect of competitor relatedness on their mortality and injury rates was also significant. Our approach in including injured males in the analysis was justified by previous findings showing that 70% of paralysed males were dead after 24 h and that the great majority of these individuals were reproductively dead (Zenner *et al.*, 2014). Moreover, at the time of dissection, it was impossible to determine the duration of interaction among competitor males as accurately as in the experiments in hanging drops.

The effect of competitor group size on male mortality was positive in small group sizes (experiment 2) and negative in large group sizes (experiment 3). This might be due to the different experimental conditions regarding density of competitors (small vs. large number of competitors), fighting area (a round hanging drop vs. a relatively large elongated host) and lastly the absence/presence of females. Alternatively, these results together confirm the theoretical prediction of a domed-shaped relationship between the intensity of fighting and competitor density: increased densities of competitors lead to an increase in aggressive interactions, but high competitor densities also increase significantly the cost of fighting (Murray, 1987; Reinhold, 2003). The weaker effect of relatedness in the more natural insect infection experiment can be explained by the low mortality rates at high male densities due to the high cost of competition in the presence of many competitors. Put simply, when fighting is not a good strategy then the effect of relatedness weakens. Nonetheless, mortality and injury rates of competing males, for a given male group size, were higher in groups of less related individuals. Alternatively, the presence of adequate number of females could reduce competition (Murray & Gerrard, 1984; Murray, 1987, 1989; West *et al.*, 2001; Reece *et al.*, 2007), but mortality rates in our experiments were not influenced by the availability of resources competed for (number of females).

Influence of relatedness on mortality

Theory predicts that when dispersal is limited, that is in viscous populations, the effect of kin is cancelled by the effect of increased competition; in essence, it does not pay to be kinder to one of your relatives at the expense of another one (Queller, 1992, 1994; Taylor, 1992a,b;

Wilson *et al.*, 1992; West *et al.*, 2002). These theoretical expectations are largely confirmed in some arthropods such as parasitoid wasps and mites wherein closely related males show extreme aggression while competing for mates (Hamilton, 1979; West *et al.*, 2001; Innocent *et al.*, 2011). In cases where competing individuals are able to recognize kin, relatedness is expected to have a dome-shaped effect with more conflict expected in conditions of intermediate relatedness, that is a mixture of related and nonrelated individuals, which can be explained in the framework of spiteful behaviour (Reinhold, 2003; Gardner & West, 2004; Gardner *et al.*, 2004; Sato *et al.*, 2013). Intermediate relatedness of male competitors in *S. longicaudum* is likely; steinerematid nematode IJs may aggregate during dispersal, at least for some distance (El-Borai *et al.*, 2011; Shapiro-Ilan *et al.*, 2014). Such group dispersal is favourable for IJs: invading a host as part of a group confers fitness advantage to individual IJs, because establishing an infection is facilitated when multiple IJs invade a host, overcoming its defences (Peters & Ehlers, 1997; Koppenhöfer *et al.*, 2006). Thus, one host can be infected either by a group of IJs originating from one cadaver or from multiple groups of IJs originating from multiple cadavers. Nonetheless, our experiments failed to find a dome-shaped influence of relatedness on aggression. Thus, killing less related competitor males cannot be viewed as freeing up resources for more closely related ones as is expected from Hamiltonian spiteful behaviour (Gardner & West, 2004).

The monotonic effect of relatedness on aggression in the nematode system shows that any benefits of being less aggressive to relatives override the costs of competing with them, even if interactions are on a local scale. One explanation of this finding is that individuals within hosts can be viewed as large groups within which multiple generations develop, a condition that could erode the strict population structure and thus weaken the genetic association of individuals within the host cadaver at any given time (Lehmann *et al.*, 2009). Moreover, entomopathogenic nematodes have a clumped and patchy distribution with patches going extinct and recolonized as a result of limited IJ dispersal and availability of immobile hosts inhabiting soil (Stuart & Gaugler, 1994; Blouin *et al.*, 1999; Stuart *et al.*, 2006). Even more, population structure of nematode parasites is more likely to follow a stepping-stone model driven by the availability of hosts suitable for reproduction (Dybdahl & Lively, 1996; Wielgoss *et al.*, 2008). These patterns of alternating/budding viscosity, transient metapopulations and successive bouts of periodic dispersal over short distances allow the beneficiaries of cooperation to compete for resources against nonrelatives and consequently promote the evolution of cooperative behaviour (Goodnight, 1992; Taylor, 1992a; Wilson *et al.*, 1992; Queller, 1994; Taylor & Irwin, 2000; Irwin & Taylor, 2001).

Kin recognition

Our experiments suggest that kin recognition in these nematodes has at least some genetic components rather than being strictly environmental. In the hanging drop experiments, adult male nematodes developed individually from IJs in separate haemolymph drops, and then, they were placed in groups of freshly prepared haemolymph drops. Thus, their kin recognition ability relies purely on cues deriving directly from them (surface or excreted), which indicates a self-referent phenotype matching mechanism (Penn & Frommen, 2010). In fact, chemical communication via ascarosides is well established and olfaction is well studied in steinernematid nematodes (Hallem *et al.*, 2011; Choe *et al.*, 2012; Dillman *et al.*, 2012). In the cadaver experiment, however, juveniles mature to adults in a mix of bacteria/bacterial compounds and so all share the same 'nest' smell. Sharing a common environment among individuals who are able to recognize kin based on genetic cues could lead to imperfect kin recognition with important consequences for the evolution of altruistic/cooperative behaviour (Manning *et al.*, 1992; Pfennig, 1997). On a mechanistic level, the lower mortality observed in the cadaver experiment can be explained by the balance between the costs of accepting undesirable nonrelatives opponents and rejecting desirable closer relatives, which is determined by discrimination abilities of the actors (Reeve, 1989). In the case of entomopathogenic nematodes, imperfect kin recognition could prompt competitors to be more tolerant rather than aggressive at higher relatedness (Segoli *et al.*, 2009).

Genetic kin recognition systems are not evolutionarily stable and drive themselves to extinction because individuals bearing common phenotypic cues (markers) enjoy higher fitness eventually leading to the fixation of the common marker until this marker no longer bears an indicator of kinship (Crozier, 1986; Rousset & Roze, 2007). However, these restrictions can be relaxed in spatially structured populations (Axelrod *et al.*, 2004; Jansen & van Baalen, 2006; Rousset & Roze, 2007). Reduced dispersal and low recombination could lead to indirect benefits of individuals bearing rare markers because they are interacting with more cooperative social partners and thus having higher fitness than individuals bearing common markers. This could protect the marker diversity that is needed for genetic kin recognition to be maintained, giving at least a transient benefit to marker-based helping (Grafen, 1990). Moreover, genetic kin recognition can be maintained by balancing selection imposed by host–parasite interactions (Crozier, 1986; Gardner & West, 2007), and this has been demonstrated in other nonvertebrate systems (Giron & Strand, 2004; Martin *et al.*, 2011; Jongepier & Foitzik, 2015). Future research should be focused on the (likely chemical) cues that nematodes use to recognize their relatives.

Conclusion

Alternative hypotheses regarding local competition between relatives for access to limited resources have been tested in only a few experimental systems (West *et al.*, 2001; Gardner *et al.*, 2004; Giron *et al.*, 2004; Innocent *et al.*, 2011; Sato *et al.*, 2013). Our study shows that males of the entomopathogenic nematode *S. longicaudum* that engage in lethal fights with their rivals recognize and are less aggressive towards their kin leading to lower mortality and injury rates. This monotonic effect of relatedness on aggression in our nematode system confirms that cooperation can be even more widespread than thought in patchily distributed organisms with low dispersal and that genetic kin recognition systems can be even selected for under these circumstances. Furthermore, our results are in agreement with theoretical studies showing the conditions for the evolution of marker-based harming behaviour in structured populations are not so stringent as thought (Lehmann *et al.*, 2009). This nematode system can provide interesting new opportunities for studying kin recognition, competition and cooperation-related phenomena.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Supplementary material.

Data deposited at Dryad: doi: 10.5061/dryad.8428t

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