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Interference competition in entomopathogenic nematodes: male *Steinernema* kill members of their own and other species

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ABSTRACT

There is evidence of competition within and between helminth species, but the mechanisms involved are not well described. In interference competition, organisms prevent each other from using the contested resource through direct negative interactions, either chemical or physical. *Steinernema* spp. are entomopathogenic nematodes; they enter a living insect host which they kill and consume with the aid of symbiotic bacteria. Several studies have demonstrated intra- and interspecific competition in *Steinernema*, mediated by a scramble for resources and by incompatibility of the bacterial symbiont. Here we describe a mechanism by which male *Steinernema* may compete directly for resources, both food (host) and females, by physically injuring or killing members of another species as well as males of their own species. A series of experiments was conducted in hanging drops of insect haemolymph. Males of each of four species (*Steinernema longicaudum*, *Steinernema carpocapsae*, *Steinernema kraussei* and *Steinernema feltiae*), representing three of the five phylogenetic clades of the genus, killed each other. Within 48 h, up to 86% of pairs included at least one dead male, compared with negligible mortality in single male controls. There was evidence of intraspecific difference: one strain of *S. feltiae* (4CFMO) killed while another (UK76) did not. Males also killed both females and males of other *Steinernema* spp. There was evidence of a hierarchy of killing, with highest mortality due to *S. longicaudum* followed by *S. carpocapsae*, *S. kraussei* and *S. feltiae*. Wax moth larvae were co-infected with members of two *Steinernema* spp. to confirm that killing also takes place in the natural environment of an insect cadaver. When insects were co-infected with one infective juvenile of each species, *S. longicaudum* males killed both *S. feltiae* UK76 and *Steinernema hermaphroditum*. Wax moths co-infected with larger, equal numbers of *S. longicaudum* and *S. feltiae* UK76 produced mainly *S. longicaudum* progeny, as expected based on hanging drop experiments.

1. Introduction

Competition amongst parasites, as amongst free-living species, occurs by two broad means, exploitation and interference (Dobson, 1985). In exploitation competition, individuals compete for the same resources; this is widespread where parasites of the same species congregate at their preferred site and compete for limited resources as populations increase (Kennedy, 1975); exploitation competition may also occur between parasite species with overlapping niches (Dobson, 1985). In interference competition, organisms prevent each other from using the contested resource through direct negative interactions. A special form of competition exclusive to parasites is host-mediated, where a parasite triggers an immune response in the host, or induces other changes in the host, that negatively affects a second species (Behnke et al., 2001; Cox, 2001). Negative interactions between species are a significant phenomenon in the ecology of parasites, occurring in a broad taxonomic range of both parasites and hosts (Halvorsen, 1976), and the importance of species interactions in structuring parasite communities is acknowledged (Esch et al., 1990; Poulin, 2001), but the exact mechanisms by which parasites interact – through competition for resources, through host mediated effects or by direct interaction – are not well known.

Direct negative interactions between organisms may be chemical or physical (Schoener, 1983). There is some evidence for the involvement of chemicals – a so-called "crowding factor" in intraspecific interactions amongst cestodes (Roberts, 2000). Chemical interactions are ubiquitous in microparasites – for example, bacteriocins are produced by bacteria to kill closely related species (Riley and Wertz, 2002; Mideo, 2009). Due to the ease of experimentation, invertebrate hosts offer greater opportunities than vertebrates for researching mechanisms of interaction between parasites. Harmful physical interactions occur amongst trematodes

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in the snail intermediate host: the rediae of many echinostome species attack and consume rediae and sporocysts of other trematode species, thereby eliminating subordinate species from the snail (Lie et al., 1968; Lim and Heyneman, 1972; Hechinger et al., 2011). In solitary species of parasitoid insects a host can support only one individual. Most solitary endoparasitoids produce first instars with enlarged mandibles; these larvae move through the host haemocoel and locate competitors which they fight with their mandibles until one of the opponents is killed (Strand, 2002; Harvey et al., 2013).

Similar to parasitoid insects, the entomopathogenic nematodes (EPNs), *Steinernema* spp., utilise the resources provided by a killed insect host, which they may contest by various means. Intraspecific competition for host resources has been documented from experimental infections (Zervos et al., 1991; Selvan et al., 1993; Koppenhöfer and Kaya, 1995); such resource competition is inevitable, regardless of initial infection level, as steinernematids multiply within the host cadaver. In experimental infections where two *Steinernema* spp. co-infect the one host individual, varied outcomes in terms of the reproductive output of each species have been reported. These range from little effect to severe reduction, depending on the species combination and factors such as relative inoculum size; however, normally one species predominates in the emerging progeny (Kondo, 1989; Koppenhöfer et al., 1995; Sicard et al., 2006; Puza and Mracek, 2009, 2010; Bashey et al., 2011, 2012). Both exploitation and interference competition are implicated in the dominance of one species over another. *Steinernema* spp. are mutualistically associated with bacteria of the genus *Xenorhabdus* which contribute to the killing of the host and its conversion into suitable food for the nematodes (Forst et al., 1997). The association between nematodes and parasites is quite specific; each species of *Steinernema* associates with a single species of *Xenorhabdus*, although one species of *Xenorhabdus* may associate with more than one *Steinernema* sp. (Adams et al., 2006). *Steinernema* spp. differ in the extent to which they rely on their own symbiont and tolerate those of other species (Akhurst, 1983; Akhurst and Boemare, 1990; Sicard et al., 2003, 2004, 2005). Koppenhöfer et al. (1995) proposed that the superiority of *Steinernema glaseri* over *Steinernema carpocapsae* in co-infected hosts was due both to *S. glaseri*'s faster development rate and to its less specific relationship with its bacterial symbiont, allowing it to develop on the bacterial symbiont of its competitor. The relative numbers of bacteria carried by the infective juveniles (IJs) of each species (Sicard et al., 2006) and the ability of the symbionts to produce bacteriocins (toxins that suppress other related strains of bacteria) (Hawlena et al., 2010; Bashey et al., 2012) may also affect the outcome of the interaction between nematode species by favouring one symbiont over the other. Experimentation with different species combinations has supported the contention that both exploitation and symbiont-mediated interference competition may contribute to the greater success of one species over the other (Sicard et al., 2006; Bashey et al., 2011, 2012). We have recently shown that nematodes are also capable of inflicting direct injury on each other: male *Steinernema longicaudum* coil around and squeeze each other, resulting in paralysis and death (Zenner et al., 2014).

In *Steinernema*, transmission is achieved by the IJ which actively seeks out insect hosts in soil and other cryptic habitats. IJs carry cells of their *Xenorhabdus* symbiont in a specialised structure of the intestine, the receptacle (Kim et al., 2012). Once in the host haemocoel (blood cavity) the symbiont is expelled from the IJ and begins to multiply (Ciche et al., 2006). Death of the host normally ensues within 2 days. The IJs recommence development to adults, which are amphimictic males and females in a slightly female-biased sex ratio (Poinar, 1990; Alsaiyah et al., 2009). An exception is *Steinernema hermaphroditum* whose morphological females are functionally hermaphroditic and males are rare (Griffin et al., 2001). Eggs are initially laid but later, juveniles hatch within the mother and consume her, resulting in her death (Poinar, 1990; Baliadi et al., 2004). *Steinernema* can have several generations within the host. As the host resources are depleted IJs form, are colonised by the symbiont (Martens et al., 2003) and disperse in search of fresh hosts. More than 100,000 IJs can be produced from a single host cadaver (Dutky et al., 1964; Lindegren et al., 1993; Shapiro-Ilan et al., 2002). They will thus experience intense competition for food resources, whether the host is infected with one or more species. Crowding is more likely to be a problem in subsequent generations rather than the founding one. Therefore it is advantageous to kill competitors of the founding generation to reduce competition for host resources amongst progeny in subsequent generations, while killing amongst these progeny would be less profitable. As expected, killing amongst *S. longicaudum* males was stage-specific, being expressed mainly in those that had passed through the IJ stage i.e. first generation colonists of the host (Zenner et al., 2014). Therefore, experiments described here concentrate on first generation, IJ-derived adults.

Here we test whether males of species of *Steinernema* other than *S. longicaudum* also kill each other. In EPNs, the killing of rivals secures access not only to females but also to food resources for progeny. Killing members of other species that compete for the finite food resources of the insect cadaver would also be beneficial. Therefore we also extend the investigation to interspecific interactions.

2. Materials and methods

2.1. Source and maintenance of nematodes

Six strains of five species of *Steinernema* were used in experiments, including members of three of the five clades of the genus (Table 1). Nematodes were routinely maintained using standard procedures by passage through late instar *Galleria mellonella* (wax moth) larvae (Kaya and Stock, 1997). Both routine culture and rearing in haemolymph were carried out at a temperature appropriate for each species: 27 °C for tropical species (*S. longicaudum* and *S. hermaphroditum*); for temperate species either 20 °C (*Steinernema feltiae* and *S. carpocapsae*), or 15 °C for the cold-adapted *Steinernema kraussei*. For experiments where members of tropical and temperate species were tested against each other, they were all reared and tested at an intermediate temperature (23 °C).

2.2. In vitro: experiments in hanging drops of insect haemolymph

Nematodes used in experiments were reared in hanging drops of insect haemolymph, so that their social experience could be controlled. Cultures were initiated using IJs from *G. mellonella* culture. IJs were surface sterilised using hyamine and transferred to a hanging drop of *G. mellonella* haemolymph (Kaya and Stock, 1997). Symbiotic bacteria released by the IJ multiply in the medium, providing suitable nutrition for the nematodes and suppressing contaminating micro-organisms (Forst et al., 1997). Adults developed in 2–6 days depending on species. Unless otherwise stated, solitary-reared nematodes with no prior social experience were used in experiments.

2.2.1. Intraspecific killing in vitro

Intraspecific killing was tested for five of the six nematode strains, using pairs of individually reared males with no prior social experience. *Steinernema hermaphroditum* was not included, as very few males are found in this species (Griffin et al., 2001). One adult male was transferred into a drop containing a second adult male of

Table 1
Steinernema strains used in experiments, identity of associated *Xenorhabdus* spp. and phylogenetic clade of both symbiont partners.

Steinernema spp. and strains	Clade[a]	Associated *Xenorhabdus* spp.	Clade[b]	Source
Steinernema longicaudum CB2B	V	*Xenorhabdus ehlersii*	X-I	CABI, UK
Steinernema hermaphroditum T87	V	*Xenorhabdus griffiniae*	X-I	Seram, Indonesia; Own collection, C. Griffin, National University of Ireland (NUI) Maynooth, Ireland
Steinernema carpocapsae All	II	*Xenorhabdus nematophila*	X-II	Reading University, UK
Steinernema kraussei	III	*Xenorhabdus bovieni*	X-III	Becker Underwood, UK
Steinernema feltiae 4CFMO[b]	III	*X. bovieni*	X-III	County Mayo, Ireland; Own collection, C. Griffin, NUI Maynooth, Ireland
S. feltiae UK76 [a]	III	*X. bovieni*	X-III	Becker Underwood, UK

[a] Nadler et al. (2006).
[b] Tailliez et al. (2010).

the same species using either a platinum wire or a microcapillary tube with an attached aspirator. Males of the same age were left on their own, to control for natural mortality. There were 19–51 pairs and 14–55 singletons per species (Table 2). Nematodes were observed 24–48 h later and the condition of each male was recorded.

The above experiments were conducted with nematodes that had been reared in isolation and had had no prior social experience before being tested. Rearing in social isolation may affect nematode behaviour (Rose et al., 2005) and social conditions also affect the expression of fighting and aggressive encounters in animals (Murray, 1987, 1989; Enquist and Leimar, 1990). Some additional experiments were carried out to confirm that killing is not restricted to very limited rearing or testing conditions. Zenner and Griffin (unpublished observations) showed that *S. longicaudum* also killed when previously mated and when in mixed sex social groups. We tested whether these findings also extend to *S. carpocapsae*, the most widely studied member of the genus. One individually reared *S. carpocapsae* male and one female were paired for 24–48 h. Successful mating was subsequently confirmed by progeny production. Pairs of mated *S. carpocapsae* males ($n = 13$) were then put together (without a female present) and observed after 24 h. In a separate experiment, *S. carpocapsae* was reared in mixed sex social groups. Hanging drops ($n = 34$) were inoculated with varying numbers of IJs, up to approximately 25. The drops were observed after 5 days. The numbers of males and females present, and whether they were alive or dead, were recorded.

2.2.2. Do males kill members of another species in vitro?

A single adult male of one species was transferred into a haemolymph drop containing an adult male or female of another species. For interspecific male–male contests, three species (*S. longicaudum*, *S. carpocapsae* and *S. feltiae* UK76) were tested. Contests were staged in a haemolymph drop in which one of the males (resident) had developed; each species combination was tested with each species both as resident and as intruder. There were thus six pair-wise combinations (treatments), shown (with n) in Table 3. As controls, single males of each species were left in the haemolymph drop in which they had developed ($n = 57$ total for all three species), and additional single males were transferred into a haemolymph drop vacated by a male of the other species ($n = 50$ total for all combinations).

In order to rule out attempted copulations in the absence of females as the cause of killing – the so-called "prisoner effect" (Bailey and Zuk, 2009) – we tested whether an *S. longicaudum* male would kill a male of another species even when a female of his own species was present. A male and a female *S. longicaudum* that had been reared individually were transferred together into a drop containing either a male or a female *S. feltiae* UK76. For comparison, only a male *S. longicaudum* was transferred.

When testing whether males kill females of another *Steinernema* sp., the target female (*S. hermaphroditum*, *S. longicaudum*, *S. carpocapsae* or *S. feltiae* UK76) was left in the haemolymph drop in which it had developed with its native symbiont, and a male (*S. longicaudum*, *S. carpocapsae* or *S. feltiae* UK76) that had been reared in isolation was transferred into that drop. Combinations tested ($n = 20–57$) are shown in Table 4. Controls were single females left alone in their own drops ($n = 40–70$). As an additional control, a female of one species was added to the drop of a female of another species (*S. longicaudum*, *S. carpocapsae* in reciprocal transfers, $n = 9$ or 18).

2.2.3. Lifespan of male and female Steinernema reared alone in hanging drops

Some (see Table 5 for n) of the *S. longicaudum*, *S. carpocapsae* and *S. feltiae* UK76 that had been reared in isolation from IJs (as controls) were kept indefinitely and time of death was noted.

2.3. In vivo: experiments in wax moth larvae

2.3.1. Nematode mortality in insects infected with two species of Steinernema

Based on the results of hanging drop experiments, we predicted that *S. longicaudum* males should kill female *S. hermaphroditum*. Wax moth larvae were infected with either a single *S. longicaudum* IJ and a single *S. hermaphroditum* IJ (41 insects) or a single

Table 2
Outcome of dyadic contests between males of the same *Steinernema* spp. Mortality measured at 24 h after two *Steinernema* males of the same species were placed together, compared with mortality in single males. Within the strain, the total number (n) of nematodes dead when in pairs was compared with a single male control by Chi-square or Fisher's Exact test. For the comparison between strains, the number of pairs with at least one male dead was compared (Chi-square = 47.81, degrees of freedom (d.f.) = 4, $P < 0.001$). The values followed by different letters differ by pairwise 2×2 Chi-square tests employing a Bonferroni correction ($P < 0.005$).

Clade	*Steinernema* spp./strains	n dead/n total		Comparison of pair versus single	
		Pair	Single	χ^2,1 d.f.	P
V	*Steinernema longicaudum*	44/51 (86%) a	0/19 (0%)	12.880	<0.001
II	*Steinernema carpocapsae*	37/47 (79%) ab	0/18 (0%)	10.580	=0.001
III	*Steinernema kraussei*	31/51 (61%) bc	1/49 (2%)	15.930	<0.001
III	*Steinernema feltiae* 4CFMO	24/48 (50%) c	1/55 (2%)	13.602	<0.001
III	*S. feltiae* UK76	1/19 (5%) d	0/14 (0%)	–	>0.9

Table 3
Outcome of dyadic contests between males of different *Steinernema* spp. and strains. Number (*n*) of *Steinernema* males dead 48 h after an intruder of one species was placed into the hanging haemolymph drop containing a male of another species (resident). Mortality of single male controls was <1% (total *n* for all treatments = 107).

Resident male	Intruder male	*n*	Outcome		
			Resident dead	Intruder dead	Neither dead
Steinernema feltiae UK76	*S. longicaudum*	20	16	1	3
Steinernema longicaudum	*S. feltiae* UK76	20	0	17	3
S. feltiae UK76	*S. carpocapsae*	15	7	2	6
Steinernema carpocapsae	*S. feltiae* UK76	15	2	9	4
S. longicaudum	*S. carpocapsae*	20	0	20	0
S. carpocapsae	*S. longicaudum*	20	12	4	4

Table 4
Outcome of dyadic contests involving *Steinernema* males and females of different species. Mortality of resident *Steinernema* female (or hermaphrodite, *Steinernema hermaphroditum*) 24–48 h after a male or female of a different *Steinernema* sp. (intruder) was introduced into the drop of haemolymph in which the resident had developed, together with the probability that the observed mortality was different from that of controls where the female was alone (Chi-square or Fisher's Exact test[a]).

Intruder sex	Intruder *Steinernema* spp. and strains	Resident *Steinernema* female or hermaphrodite	*n* residents dead/*n* total	χ^2 (1 d.f.)	*P*
Male	*Steinernema longicaudum*	*S. feltiae* UK76	45/57 (78.9%)	81.724	<0.001
		S. carpocapsae	19/21 (90.5%)	59.850	<0.001
		Steinernema hermaphroditum	42/48 (87.5%)	55.883	<0.001
	Steinernema carpocapsae	*S. feltiae* UK76	10/20 (50%)	–	<0.001
		S. longicaudum	3/34 (8.8%)	–	0.104
	Steinernema feltiae UK76	*S. carpocapsae*	2/33 (6%)	–	0.56
		S. longicaudum	4/28 (14.3%)	–	0.023
Female	*S. longicaudum*	*S. carpocapsae*	0/9 (0%)	–	1
	S. carpocapsae	*S. longicaudum*	2/18 (11.1%)	–	0.107
Male + female	*S. longicaudum*	*S. feltiae* UK76	9/12 (75%)	–	<0.001
None	None	*S. feltiae* UK76	1/70 (1.4%)	–	–
		S. carpocapsae	1/53 (1.9%)	–	–
		S. hermaphroditum	3/40(7.5%)	–	–
		S. longicaudum	1/69 (1.4%)	–	–

n, number; d.f., degrees of freedom.
[a] Two tailed Fisher's Exact test was used where an expected value <5 was found in the Chi-square test.

Table 5
Average lifespan (days) of male and female *Steinernema* spp. in hanging drops of *Galleria mellonella* haemolymph at 23 °C. Nematodes were reared and maintained individually.

Steinernema spp. and strains	Mean lifespan in days (±S.E.); *n*	
	Female	Male
Steinernema longicaudum	25.5 (±1.73); 63	19.9 (±4.74); 14
Steinernema carpocapsae	24.4 (±2.60); 33	21.4 (±4.12); 26
Steinernema feltiae UK76	18.6 (±2.52); 19	not tested

n, number tested.

S. longicaudum IJ and a single IJ of *S. feltiae* UK76 (45 insects) to determine whether killing occurs in vivo. The IJs were placed on a piece of moistened filter paper lining a 1.5 ml microcentrifuge tube with a hole pierced in the lid. One *G. mellonella* larva was placed into the tube. Control infections were set up for *S. hermaphroditum* and *S. feltiae*, where *G. mellonella* larvae were infected with a single IJ. Tubes were stored at 23 °C for 4 days, after which cadavers were removed and dissected. The sex and condition of each adult nematode were recorded. In the case of the *S. longicaudum*–*S. feltiae* infections, the species of the adults were also identified, where possible, based on morphological characteristics.

2.3.2. Survival and reproductive output of S. longicaudum and S. feltiae UK76 in insects with multiple IJs of one or both species

Based on the results of the hanging drop experiments, we also predicted that *S. longicaudum* males would kill *S. feltiae* males and females. The two species have a similar, slightly female-biased sex ratio in insects (Alsaiyah et al., 2009). Wax moth larvae were infected with either *S. longicaudum*, *S. feltiae* or a combination of both. Each *G. mellonella* larva was placed, individually, into a 16 mm diameter well in a 24 well plate. Each well was lined with filter paper (15 mm diameter). Nematode suspension (50 µl containing approximately 50 IJs of either *S. longicaudum* or *S. feltiae*, or 25 IJs of each species) was pipetted onto the filter paper in each well. After 4 days at 23 °C, insect larvae were dissected and the number of adult nematodes alive and dead within each insect was recorded. The experiment was conducted twice, with eight or seven cadavers per treatment dissected, respectively. In the second run of the experiment, additional cadavers were included for each treatment and left to allow the nematodes to reproduce. These cadavers were placed in White traps and IJs were harvested until emergence ceased. Sample counts were carried out on the harvested IJs to estimate the yield. There were three White traps per treatment, each with three cadavers. The IJs emerging from insects exposed to a single nematode species were assumed to be of that species. For the two-species infection, a sample of IJs was taken from each of the White traps for species identification. IJs were reared to adult in hanging drops of haemolymph (one IJ per drop), and identified based on morphology and cross-breeding. While still alive, each adult was assigned provisionally to species based on morphological characters. A known member of this species, of the opposite sex, was then added. These mating partners had also been reared individually in haemolymph. Where progeny were produced, the identity was confirmed. As none of the suspected *S. feltiae* from the first two replicate White traps produced progeny, an alternative strategy was adopted for the third replicate. Males were killed, examined under high powered magnification and assigned to species based on morphological criteria. All unknown females were challenged with a male *S. longicaudum*. Progeny production identified the female as *S. longicaudum*, while

females that died within 24 h, without producing offspring, were scored as *S. feltiae*.

2.4. Statistics

Statistical tests were carried out using Minitab16.0. Incidence of mortality in hanging drops of different treatments was compared by cross tabulation using Chi-square. Fisher's Exact test (two-tailed) was used where one or more expected values was less than 5. A Bonferroni correction was applied for multiple pair-wise comparisons of mortality between nematode strains. Continuous data were compared using General Linear Model (GLM) (for two-way analysis of an unbalanced data set) or one-way ANOVA, followed by a Tukey's test where significance was detected. In the in vivo experiment, the proportion of dead nematodes was transformed by arc-sine square root prior to analysis. Significance was accepted at $P < 0.05$.

3. Results

3.1. In vitro experiments: killing in hanging drops

3.1.1. Intraspecific male–male killing in four Steinernema spp.

When two male nematodes of the same strain were placed together in a drop of haemolymph, high mortality (one male dead in 50–86% of the pairs) was recorded 24 h later in four of the five strains tested, representing four species (Table 2). Mortality in single male controls was negligible (0–2%), with a highly significant difference from mortality in pairs of the same strain except in the case of *S. feltiae* UK76 (Table 2). There was a highly significant difference amongst the five strains in the proportion of pairs with at least one male dead (Chi-square = 47.81, d.f. = 4, $P < 0.001$), being highest (86%) in *S. longicaudum* and lowest in *S. feltiae* (Table 2). Within *S. feltiae*, there was one dead male in 50% of pairs for strain 4CFMO and only 5% for strain UK76, a value that did not differ from that of single male controls (Table 2).

Male *S. carpocapsae* that had previously mated killed each other, with one dead male in 9/13 pairs (69%), a rate similar to that of virgin males (79%; Table 2). Mating status of protagonists did not affect the probability of mortality (Fisher's Exact test, $P > 0.05$).

Steinernema carpocapsae were reared from IJs in mixed sex social groups of various sizes. Where only one male was present (together with 0–5 females), it remained alive in all cases ($n = 29$). Most multi-male groups (2–9 males per group; $n = 15$) contained at least one dead male and more than 50% had just one live male. Most of the multi-male drops also contained one or more females. A total of only 3/51 females (6%) were dead across all multi-male groups, compared with 29/63 males (46%) in the same groups; a highly significant difference (Chi-square = 22.501, d.f. = 1, $P < 0.001$). The 46% death rate for males in these mixed social groups is similar to the 40% death rate in pairs of naive nematodes (37/94 dead males in the 47 pairs; Table 2); Chi square = 0.689, d.f. = 1, $P = 0.407$.

3.1.2. Steinernema males kill males of other species

When a male *S. longicaudum* and a male *S. feltiae* were placed together, 33/40 (82.5%) of the *S. feltiae* and only 1/40 (2.5%) of the *S. longicaudum* males died (Table 3). Male *S. feltiae* (16/30; 53.3%) were also killed by *S. carpocapsae*, of which a small number (4/30; 13.3%) also died in this pairing. In both of these species combinations, the outcome was similar irrespective of which male was the resident (Table 3). Transferring an *S. longicaudum* female together with the *S. longicaudum* intruder male into a resident *S. feltiae* male's drop did not affect the outcome: 4/5 (80%) of the *S. feltiae* males died, a value that did not differ from the outcome with only a male intruder ($P > 0.05$, Fisher's Exact test; data not shown).

When *S. longicaudum* and *S. carpocapsae* males were paired, 32/40 (80%) of the *S. carpocapsae* died compared with 4/40 (10%) of *S. longicaudum* (Table 3). Residency affected the outcome for *S. carpocapsae* (Fisher's Exact test, $P = 0.0033$), but not for *S. longicaudum* ($P > 0.05$). *Steinernema carpocapsae* suffered higher mortality when it was an intruder in an *S. longicaudum* drop than when it was resident in its own drop (Table 3).

Killing by the three species was compared. There was a highly significant difference between species in the proportion of opponents that they killed (Chi-square = 91.281, d.f. = 2, $P < 0.001$). *Steinernema longicaudum* killed 65/80 (81.3%), *S. carpocapsae* killed 20/70 (28.6%), and *S. feltiae* killed 5/70 (7.1%) of opponents (data for both opposing species combined, Table 3).

Death of the single male controls was negligible, whether they were in the haemolymph drop in which they had been reared, or in a drop in which a male of another species had developed and subsequently had been removed. The only single male that died was a *S. carpocapsae* male in a drop previously occupied by an *S. longicaudum* male ($n = 15$).

3.1.3. Steinernema males kill females of other species

Mortality of female *Steinernema* on their own (controls) was low, with a maximum of 7.5% recorded for *S. hermaphroditum* (Table 4). However, when a male of another species was placed with her for 24–48 h, high mortality was recorded in several instances. Females that died in the presence of a male often had a damaged and/or ruptured cuticle. When the intruding male was *S. longicaudum*, 79–90.5% of females died. This level of mortality differed from that of the relevant single-female control in each case (*S. feltiae*, *S. carpocapsae* and *S. hermaphroditum* Chi-square test, d.f. = 1; $P < 0.001$, Table 4). When the intruding male was *S. carpocapsae*, 50% of *S. feltiae* UK76 females died ($P < 0.001$) but the mortality of *S. longicaudum* females did not differ from that of the controls. In the presence of an *S. feltiae* UK76 male, mortality of *S. carpocapsae* females did not differ from controls, while 14% of *S. longicaudum* died ($P < 0.05$). Intruding females of *S. longicaudum* or *S. carpocapsae* did not result in the death of resident females (Table 4), nor did any of the intruding females die.

Males of the three *Steinernema* spp. differed in the extent to which they killed females; in general, the highest female mortality was due to *S. longicaudum* males and the lowest was recorded with males of *S. feltiae* UK76. We compared the mortality due to the various males for each species of resident female separately. More *S. feltiae* UK76 females died when with a male *S. longicaudum* than when with a male *S. carpocapsae* (Chi-square test, $\chi^2 = 6.08$, d.f. = 1, $P = 0.014$). For *S. carpocapsae*, more females died in the presence of a male *S. longicaudum* than a male *S. feltiae* UK76 (Chi-square test, $\chi^2 = 38.48$, d.f. = 1, $P < 0.001$,). However, for *S. longicaudum* females, mortality was low ($\leqslant$14%) with a male of either *S. feltiae* or *S. carpocapsae*, with no difference detected between the two species (Fisher's Exact test, $P > 0.05$).

When an *S. longicaudum* male and female were transferred together into a drop containing a single *S. feltiae*, the resident *S. feltiae* female died in 75% of cases, a rate similar to that observed when a *S. longicaudum* male was transferred on its own (79%) (Table 4). The presence of a *S. longicaudum* female together with the *S. longicaudum* male did not affect the probability of death for *S. feltiae* females ($P > 0.05$, Fisher's Exact test).

3.1.4. Lifespan of male and female Steinernema reared alone in hanging drops

The mean lifespan of nematodes reared and maintained alone in hanging drops ranged from 18.6 to 25.5 days (Table 5).

3.2. In vivo: experiments in wax moth larvae

3.2.1. Nematode mortality in insects co-infected with one IJ of each of two species

Males are rare in *S. hermaphroditum*, representing 1–6% of the population, while the sex ratio of *S. longicaudum* is approximately balanced (Griffin et al., 2001; Alsaiyah et al., 2009). Therefore, where a nematode of each sex is found in an insect co-infected with one IJ of each of these two species, in the majority of cases it will be an *S. longicaudum* male with an *S. hermaphroditum*. Where a male and a female adult developed in insects infected with a single *S. longicaudum* and a single *S. hermaphroditum* IJ, a high proportion (10/13; 77%) of the females (presumed *S. hermaphroditum*) were dead. This was significantly different from the control (single *S. hermaphroditum*: 0/8 dead) and from where either one female (0/8 dead) or two females (0/40 dead) were found ($P < 0.001$ for all comparisons, two-tailed Fisher's Exact test). No dead males were found.

Of the 45 insects exposed to one IJ each of *S. longicaudum* and *S. feltiae*, 30 cadavers contained two nematodes each. Six contained two females and in each case both were alive. Four contained two males; in two of them, one of the males was dead. In both cases the dead male was identified as *S. feltiae*. Identification of the male/female pairs revealed that 7/8 *S. feltiae* (87.5%) females died in the presence of an *S. longicaudum* male but none of the 12 *S. longicaudum* females died in the presence of an *S. feltiae* male. The remaining 15 dual-exposed cadavers contained a single nematode, none of which was dead. In addition, none of the 28 *S. feltiae* recovered from singly-infected insects was dead.

3.2.2. Survival and reproductive output of S. longicaudum and S. feltiae (strain UK76) in insects infected with multiple IJs of one or both species

The dissected cadavers of each of the three treatments contained a similar number of first generation (colonising) nematodes (*S. feltiae*: 41.9 ± 1.80, $n = 15$; *S. longicaudum*: 44.9 ± 3.55, $n = 13$; Mixture: 43.7 ± 3.78, $n = 15$; ANOVA, $F_{2,40} = 0.23$, $P = 0.799$). The sex ratio did not differ between the three treatments (Chi square = 3.873, d.f. = 2, $P = 0.144$), and was slightly female-biased (female: male 1.1:1.0, $n = 1868$).

Nematode mortality varied by treatment (GLM, $F_{2,82}$, 12.73, $P < 0.001$) and by sex (GLM, $F_{1,82} = 4.03$, $P = 0.48$). Inspection of the data (Fig. 1) strongly suggests an interaction between the treatment and sex (due to the unbalanced nature of the data an interaction effect was not tested in the GLM). To further explore the variation due to sex and treatment, we subjected data from all six treatments to a one-way ANOVA ($F_{5,80} = 18.51$; $P < 0.001$). In cadavers infected with *S. longicaudum* alone, more than half (53.2%) of the males were dead, compared with only 2.9% of females. In *S. feltiae*-only cadavers, the proportion of dead males and females (5.4% and 6.6%, respectively) did not differ significantly either from each other or from the value for *S. longicaudum* females (Fig. 1). In cadavers co-infected with both species, both males and females experienced high levels of mortality (29.0% and 42.1% respectively), values greater than *S. feltiae* and for *S. longicaudum* females (Fig. 1). Dead nematodes could not be reliably identified, but in a sample of live males from co-infected hosts there were twice as many *S. longicaudum* as *S. feltiae* (110 and 52, respectively).

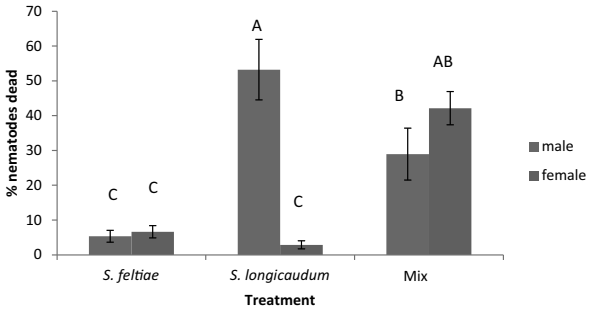

Fig. 1. Percentage of mortality (mean ± S.E.) of male and female *Steinernema* spp. dissected from *Galleria mellonella* infected 4 days previously with *Steinernema feltiae* alone, *Steinernema longicaudum* alone, or an equal mix of both species ($n = 13$ or 15 cadavers per treatment). Bars accompanied by the same letter are not significantly different (Tukey's test).

Numbers of IJs produced from the three treatments differed significantly (ANOVA $F_{2,6} = 12.65$, $P = 0.007$), with more IJs produced from the *S. longicaudum* single infection (mean ± S.E.: 235,992 ± 37,702) than from either the *S. feltiae* only (94,333 ± 8,824) or the mixed infection (81,939 ± 15,405). A sample of progeny IJ was identified as predominantly *S. longicaudum* (mean ± S.E.: 38.7 ± 12.57, 66.0% of the sample) compared with just 5 ± 1.73 *S. feltiae* (9.7% of the sample) and 12.0 ± 4.04 (24.3% of the sample) that could not be positively identified. These results were for all three replications. For the third replication alone, where the identification strategy was modified, there was a lower proportion (5.6%) of unidentified nematodes; here, 87.5% of the nematodes were identified as *S. longicaudum* and 6.9% as *S. feltiae* ($n = 72$).

4. Discussion

Here we have shown that male *Steinernema* nematodes of several species can damage and kill competitors both of their own and of other species. Negative interactions between helminth parasites may be mediated by direct competition for resources, or indirectly through host physiological and immune responses (Halvorsen, 1976; Dobson, 1985), but there are few reports of damaging physical interactions either between or within species of helminths. There is evidence of physical displacement in helminths, especially bulky cestodes inhabiting vertebrate guts (Haukisalmi and Henttonen, 1993; Behnke et al., 2001). Best documented are the interspecific interactions, including predation, between larval trematodes in snails (Lie et al., 1968; Lim and Heyneman, 1972; Hechinger et al., 2011). Homosexual rape has been documented in acanthocephalans, where cementing of the male victim's genital region effectively removed it from the reproductive population (Abele and Gilchrist, 1977; Hassanine and Al-Jahdali, 2008). While this is viewed by some as merely evidence of indiscriminate mating (Richardson et al., 1997), the fact that there is intense male–male competition for mates in the Acanthocephala (Poulin and Morand, 2000; Sinisalo et al., 2004) lends weight to the suggestion (Abele and Gilchrist, 1977) that it is sexually selected behaviour. Potentially damaging male–male interactions have been reported in free-living nematodes, although rarely. In *Oncholaimus oxyuris*, where the normal mode of insemination is by insertion of spicules through the female body wall, males were observed to "pinch" the tail region of other males and occasionally to insert the spicules into the anus or through the body wall (Coomans et al., 1988), while in all-male groups of *Caenorhabditis elegans*, males attempted mating at the excretory pore and in certain strains a copulatory plug was left there (Gems and Riddle, 2000). In neither of these reports was the behaviour believed to be sexually selected (Coomans et al., 1988; Gems and Riddle, 2000). There is indirect evidence of strong sexual selection in parasitic nematodes; for example, it is suggested that body size of males may be important in gaining and/or retaining access to females (Poulin, 1997), and the unusual distribution observed for certain oxyurids, with only one male per insect host, may also be due to competition between males (Muller-Graf et al., 2001); we have previously described male–male killing in *S. longicaudum* (Zenner et al., 2014). While

killing in *Steinernema* may have evolved through sexual selection, the same behaviour can also be used to kill members of other species that compete for the limited resources of a host insect cadaver. Here we show that *Steinernema* males do kill members of other species.

Male and female *Steinernema* nematodes lived on average between 18 and 26 days when reared alone in hanging haemolymph drops. In contrast, death occurred within 48 h (and usually within 24 h) when in the presence of a killer male. Killed males and females often displayed visible signs of injury in the form of punctured cuticles with ruptured internal organs and a shriveled, kinked appearance (A.N.R.L. Zenner, K.M. O'Callaghan, I. Dix, C.T. Griffin, unpublished observations). Although the exact cause of death is uncertain, it is clear that the lifespan of conspecific males and heterospecific males and females is severely reduced in the presence of certain *Steinernema* males. In most of our experiments, solitary-reared nematodes were placed together as adults; this would not happen in nature. However, males reared and/or tested in vitro with a range of social experience also killed. Mated *S. carpocapsae* and those reared in social groups also killed (as previously shown also for *S. longicaudum* (Zenner et al., 2014; A.N.R.L. Zenner and C.T. Griffin, unpublished observations)), and *S. longicaudum* killed both male and female *S. feltiae* even when a female of its own species was present. In vivo experiments confirmed that interspecific killing can take place under natural conditions in an insect host co-infected by two *Steinernema* spp., both at low (one IJ per species) and high (25 IJs per species) inoculum densities.

Males of each of the four species that were tested killed each other. The probability of killing varied among species, with males of *S. longicaudum* the most lethal and those of *S. feltiae* the least. In fact, whether males of *S. feltiae* strain UK76 kill is questionable, as no death was recorded among conspecific male pairs, and mortality of heterospecific females was low (significant with *S. longicaudum*, but not *S. carpocapsae* females as targets). The other strain of the species, *S. feltiae* 4CFMO, showed significant mortality in male–male conflicts (although at 50% of pairs it was lower than the other species), pointing to intra-specific as well as interspecific variation in killing amongst steinernematids. The species tested represent three of the five *Steinernema* clades, (Nadler et al., 2006), clades II, III and V (Table 1), indicating that it is an ancient character of the genus (or, less likely, that it has evolved independently several times). In additional studies in our laboratory, male–male killing was not detected in *Steinernema bicornutum* (Clade IV; $n = 21$) (Brendan Igoe, personal communication). However, failure to demonstrate killing in a species or strain of *Steinernema* may indicate that the experimental conditions were not conducive, rather than an absolute inability of the strain to kill.

Interference competition invariably operates asymmetrically (Dobson, 1985); the heterospecific male–female pairings demonstrate dominance hierarchy amongst species, with *S. longicaudum* dominating *S. carpocapsae*, and both species dominating *S. feltiae* UK76. All species where males kill are likely to be dominant in relation to *S. hermaphroditum*, where males are rare. The dominance of *S. longicaudum* over *S. feltiae* UK76 was confirmed by superior progeny production in vivo. While we have documented dominance of one species over another under our test conditions, we have not investigated the reasons for it (whether differences in motivation or physical attributes), nor the range of conditions under which this dominance would hold. For example, temperature could introduce a bias. While we used an intermediate temperature to avoid favouring one species over another, this approach may still have allowed some bias. Some steinernematid species tolerate a wider range of temperatures than others and such species would have a competitive advantage at an intermediate temperature compared with another species that possesses a narrower temperature niche breadth. For example, the niche breadth of *S. feltiae* is narrower than that of several other steinernematids (Grewal et al., 1994) and thus this species may be less competitive at an intermediate temperature than other species.

Based on the results of the in vitro experiments, and those of the single IJ co-infection experiment, we predicted that *S. longicaudum* would dominate *S. feltiae* in the IJs emerging from hosts co-infected with multiple IJs. As predicted, *S. longicaudum* constituted the majority of the emerging juveniles. While this was as expected if *S. longicaudum* killed many of the colonising *S. feltiae* females, other forms of competition (growth rates, compatibility of bacteria, etc.) may also have been involved, as postulated for other experimental co-infections (Koppenhöfer et al., 1995; Sicard et al., 2004, 2005, 2006; Bashey et al., 2012). Steinernematids do not always require their symbiont for growth and reproduction but normally do best with their natural symbiont (Akhurst, 1983; Sicard et al., 2004, 2005). The more distantly related a *Xenorhabdus* strain is to the native symbiont of a *Steinernema* sp., the less suitable it is for supporting nematode reproduction (Sicard et al., 2004, 2005). The symbionts of *S. longicaudum* and *S. feltiae* are not closely related to each other, belonging to clades X-I and X-III, respectively (Table 1; (Tailliez et al., 2010)). Further experiments would be required to ascertain whether factors other than direct killing, such as growth rate and symbiont-mediated effects, contributed to the reproductive superiority of *S. longicaudum* over *S. feltiae* in insect cadavers.

Cross-species experiments were frequently (exclusively for females, in part for males) staged in haemolymph in which the victim had developed, and hence with the victim's symbiont predominating. While a small amount of the intruder nematode's symbiont may have been transferred with it, this is unlikely to have had an adverse effect on the resident within the time-course of the experiment. Firstly, the resident nematode's bacteria would have thoroughly colonised the haemolymph over several days, giving the introduced species little opportunity to compete. Secondly, there is no evidence that short-term exposure to non-native symbionts is harmful to steinernematids. Most *Steinernema* spp. tested can develop and reproduce on at least some *Xenorhabdus* spp. other than their natural symbiont (Akhurst, 1983; Sicard et al., 2004, 2005), although for some species, symbionts that are more distantly related to their natural partner may suppress reproduction below that recorded without a symbiont, leading Sicard et al. (2004) to postulate that *Xenorhabdus* strains may produce molecules that are antagonistic to foreign nematodes. However, the negative effect described by those authors was long-term suppression of reproduction; there was no evidence of acute negative effects of *Xenorhabdus* on *Steinernema.* In our experiments also, there was negligible death of either males or females in haemolymph conditioned by the symbiont of another *Steinernema* sp., unless a competing male was present. While the effect of residency status on the outcome of the interaction between *S. longicaudum* and *S. carpocapsae* males may in part be mediated by the symbiont, residency strongly affected the outcome of intraspecific interactions for *S. longicaudum* (A.N.R.L. Zenner and C.T. Griffin, unpublished observations), suggesting that the competitors' evaluation of the resource (Enquist and Leimar, 1987; Arnott and Elwood, 2008) is at least as important.

In addition to the previously proposed mechanisms (scramble for resources and incompatibility of bacteria (Koppenhöfer et al., 1995; Sicard et al., 2004, 2005, 2006; Bashey et al., 2012)), killing of the kind reported here may help explain the dominance of one *Steinernema* sp. over another reported in other studies. Kondo (1989) reported that *S. feltiae* produced scarcely any progeny in co-infections with either *S. carpocapsae* or *S. glaseri*, in line with our finding that females of that species are killed by both *S. carpocapsae* and *S. longicaudum* (which is in the same clade as *S. glaseri*). Propagation of *S. carpocapsae* in *Spodoptera* larvae was not reduced

by *S. glaseri* in the Kondo (1989) experiments, but at equal infection levels in wax moths Koppenhöfer et al. (1995) found that progeny production of *S. carpocapsae* was reduced while that of *S. glaseri* was not. While this was explained by the authors in terms of superior growth rate and lower reliance of *S. glaseri* on its symbiont, interspecific killing may also have played a role. Interestingly, Koppenhöfer et al. (1995) noted that the proportion of females was lower in cadavers exposed to both species than in those exposed to a single species. This could be explained by the greater difficulty in detecting damaged nematodes. Puza and Mracek (2009, 2010) noted a superiority of *Steinernema affine* over *S. kraussei* in all hosts tested. As there was no difference in duration of development and both species share the same symbiont, the reason for the dominance of *S. affine* in experimental infections was unclear (Puza and Mracek, 2009, 2010). The involvement of killing in this and other interactions warrants investigation. There are thus multiple means of competition available to *Steinernema*, as in parasitoids and trematodes which use combat or predation as well as physiological suppression (Halvorsen, 1976; Strand, 2002).

For parasites of vertebrates, evidence for interactions between species comes from observed distribution patterns, as well as laboratory infections (Behnke et al., 2001; Poulin, 2001; Johnson and Buller, 2011). In contrast, virtually all of the information on interactions between species of EPNs is from laboratory infections similar to the present study (Kondo, 1989; Koppenhöfer et al., 1995; Sicard et al., 2006; Puza and Mracek, 2009, 2010; Bashey et al., 2011, 2012). Two or more species of EPN frequently occur at the same location, based on the detection of IJs (Stuart and Gaugler, 1994; Duncan et al., 2003a; Puza and Mracek, 2005; Spiridonov et al., 2007), but field data on co-infection of hosts by two species are extremely rare. Bovien (1937) noted the co-occurrence of *S. feltiae* and *S. affine* in bibionid larvae, including within the same host insect. Infrequent reporting of co-infections is not in itself evidence for the rarity of the phenomenon. Since infected hosts are usually in soil or other cryptic habitats and disintegrate within weeks, reports of natural infections of any kind, whether by one or more species, are rare (Peters, 1996). Routine monitoring of EPNs using thousands of *Diaprepes abbreviatus* weevil larvae as sentinel hosts, in areas with multiple nematode species present, has revealed co-infection with more than one species fewer than three or four times (Larry Duncan, personal communication). However, even where naturally infected hosts are recovered, it is often not the parasitic stages within it that are identified; instead, emerging IJs are either identified immediately or passed through a new host before identification (e.g. Parkman and Frank, 1992; Duncan et al., 2003b). Thus, complete suppression of a co-infecting species would be undetectable and partial suppression might also be missed. Several factors probably limit the frequency with which two species of EPN find themselves in the same host in nature, including differing host preferences, foraging strategy or preferences for certain soil properties or depths (Koppenhöfer and Kaya, 1996; Millar and Barbercheck, 2001; Spiridonov et al., 2007; Puza and Mracek, 2010; Koppenhöfer et al., 1996). Moreover, EPN populations are highly aggregated (Stuart et al., 2006), which reduces the probability of parasites co-occurring within hosts (Stuart et al., 2006), and the ephemeral nature of an infected host further reduces the probability of two species coinciding. While we have focused on the interaction between *Steinernema* spp., free-living bactivorous nematodes may also colonise insect cadavers and represent another class of competitor (Duncan et al., 2003a,b). However, the importance of interspecific competition in shaping *Steinernema* communities or evolutionary strategies is unknown (Stuart et al., 2006).

In conclusion, we have shown that killing is a widespread behaviour amongst steinernematids, that can be employed in both intra- and interspecific competition, together with other means of competition. Killing in *Steinernema* may be an adaptive response to the enclosed and transient, but high value, resource which nematodes cannot leave (Hamilton, 1979; Enquist and Leimar, 1990); if other nematode species also kill, they are most likely to be found amongst those with a similar niche. The other major genus of EPNs is *Heterorhabditis*, but as infective juveniles develop into morphologically female hermaphrodites (Poinar, 1990), killing is not expected in that genus. Other nematodes such as *Oscheius* spp. or *Caenorhabditis briggsae*, with entomopathogenic or necromenic life styles (Dillman et al., 2012), or bactivorous nematodes that may invade cadavers alone or with entomopathogens (Duncan et al., 2003a,b), may have evolved similar behaviour.

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