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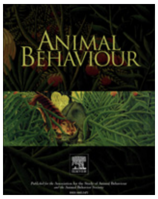

Pine weevils modulate defensive behaviour in response to parasites of differing virulence

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Grooming and avoidance of contaminated areas are among the behavioural defences employed by animals against parasites. Antiparasite defence behaviour is costly in terms of time, energy and/or food foregone and therefore animals are expected to modulate their defences depending on the risk of attack and/or the severity of the symptoms caused. We tested the hypothesis that an insect host invests more in defence against more virulent (more likely to cause death) than less virulent parasites. We tested avoidance and grooming of adult pine weevils, *Hylobius abietis*, in response to infective juveniles of two species of entomopathogenic nematodes, the more virulent *Steinernema carpocapsae* and less virulent *Heterorhabditis downesi*. Weevils avoided feeding on a substrate contaminated with *S. carpocapsae* but not *H. downesi*. Weevils also groomed more when their bodies were contaminated with *S. carpocapsae* than either *H. downesi* or water. We also made direct observations of nematodes on weevils. When equal numbers of nematodes were applied to weevils more *S. carpocapsae* than *H. downesi* moved actively on the weevil's cuticle. Thus, the differential response of weevils to the two nematode species can be explained by the weevils detecting the more aggressive behaviour of *S. carpocapsae* than *H. downesi*, which corresponds to a higher probability of death.

Most animals live in a world that is full of parasites. Since parasites frequently reduce host fitness (Price 1980), it is not surprising that animals have evolved numerous morphological, immunological and behavioural defences against them (Combes 2001). Hart (1990) recognized five behavioural strategies by which animals protect themselves against parasites, of which avoidance is the first line of defence. Behaviours employed in parasite avoidance include measures to dislodge parasites from the host animal's surface, and actions that serve to remove the host from the vicinity of the parasite. Vertebrates remove ectoparasites by grooming, and avoid parasitic or biting flies in numerous ways, including running away, grouping and microhabitat selection (reviewed in Hart 1990, 1994; Combes 2001; Moore 2002). Insects have a similar suite of behaviours to defend against their arthropod enemies: removal of ectoparasites by grooming is shown by honeybees (*Apis mellifera* and *A. cerana*) and damselflies *Ischnura verticalis* (Peng et al. 1987; Baker & Smith 1997; Boecking & Spivak 1999; Leung et al. 1999), while evasive and aggressive behaviours may help protect against parasitoid attack (Gross 1993; Gentry & Dyer 2002; Vincent & Bertram 2010).

There are many more examples of defensive reactions against arthropod than against helminth parasites, although reactions to the latter have been observed. Ungulates avoid faeces and faeces-contaminated grazing areas and this is assumed to reduce transmission of gastrointestinal parasites (Hutchings et al. 2001). Since they react similarly to faeces from parasitized and unparasitized animals, this appears to be a generalized avoidance of 'risky areas' likely to contain parasites (Cooper et al. 2000), rather than a direct response following detection of the parasites. On the other hand, fish and amphibians respond to the presence of helminths themselves: minnows, *Pimephales promelas*, learn to recognize and avoid trematode cercariae, using visual and chemical cues (James et al. 2008), while *Bufo americanus* tadpoles avoided cercariae of *Echinostoma trivolvis* without physical contact, presumably in response to chemical and/or vibrational cues (Rohr et al. 2009).

One possible explanation for the paucity of responses to helminths compared to arthropods is that helminth infective stages tend to be small relative to vertebrate hosts, making them more difficult to detect (Wisenden et al. 2009). In contrast, helminths are large relative to invertebrate hosts, and both slugs (Wilson et al. 1999) and insects respond to infective juveniles of their

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nematode parasites. Defensive reactions (grooming and evasion) to entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis* spp.) are demonstrated by various insects including ants *Solenopsis invicta* (Drees et al. 1992), termites *Zootermopsis angusticollis, Coptotermes formosanus* and *C. vastator* (Mankowski et al. 2005; Wilson-Rich et al. 2007), beetle larvae *Popillia japonica* and *Cyclocephala borealis* (Schroeder et al. 1993; Gaugler et al. 1994; Koppenhöfer et al. 2000) and cockroaches *Blattella germanica* (Appel et al. 1993). Hart (1990) stipulated two requirements that must be met for a particular behaviour to be regarded as having a parasite control function: the parasite must reduce host fitness, and the antiparasite behaviour must protect animals from infection. Entomopathogenic nematodes have the potential to reduce host fitness to zero: aided by a bacterial symbiont, they normally kill their hosts within days of infection (Kaya & Gaugler 1993). Grooming and evasion can both protect insects against these nematodes. The most commonly reported defensive behaviour by insects against entomopathogenic nematodes is grooming. Soldier *Coptotermes* that were groomed by workers to remove nematodes survived significantly longer than soldiers that were not groomed (Mankowski et al. 2005). Similarly, Japanese beetle, *Popillia japonica*, larvae brushed their bodies to remove nematodes, and this activity significantly reduced the rate of parasitism (Gaugler et al. 1994). Avoidance behaviour, including colony relocation or extension of tunnels away from contaminated areas, contributes to the difficulty of controlling ants and termites using entomopathogenic nematodes (Drees et al. 1992; Gouge 2002).

Since there are fitness costs associated with antiparasite behaviour, including direct energetic costs, indirect energetic costs associated with reduced feeding opportunities, and distraction from predator vigilance (Hart 1990), hosts are expected to adjust their behavioural response depending on the threat posed by the parasite (Hughes & Cremer 2007). This is seen, for example, in mammals which respond to biting flies more than nonbiting ones (Moore 2002). Insects have been shown to differentiate between more and less virulent strains of fungal parasites both of themselves and of their crops (Little et al. 2006; Mburu et al. 2009). There was a correlation between virulence and repellency of entomopathogenic fungi for *Macrotermes michaelseni*; the termite's response to fungal strains was directly related to the potential harm these fungi can cause the insect (Mburu et al. 2009). Fungus-growing ants *Trachymyrmex* cf. *Zeteki* removed spores of the garden parasitic fungus *Escovopsis*, but removed fewer spores that had been killed by irradiation, where the threat of parasitism was reduced (Little et al. 2006). To our knowledge, there are no previous studies on plastic responses of hosts to helminth parasites. Entomopathogenic nematodes are a promising group in which to look for associations between virulence and host defensive behaviours, since (1) it is known that insects detect and respond to their presence; (2) species vary in their virulence and this can be measured, and (3) experiments are easily conducted in the laboratory. The biology and behaviour of entomopathogenic nematodes have been extensively reviewed (e.g. Kaya & Gaugler 1993; Burnell & Stock 1999; Lewis et al. 2006). The infective juvenile, a specialized third-stage juvenile, seeks out and enters insect hosts through the mouth, anus and spiracles, and through the intersegmental membranes in some hosts. Once in the haemocoel it releases its symbiotic bacteria from its intestine and, together, the nematode–bacterial complex kills the host.

In this study, we tested the hypothesis that adult pine weevils, *Hylobius abietis*, respond to the presence of entomopathogenic nematodes by grooming and/or avoidance of a contaminated substrate; that they respond differentially to different nematode species, and that they are more likely to respond to a more virulent than a less virulent species (*Steinernema carpocapsae* and *Heterorhabditis downesi*, respectively; Girling et al. 2010). To understand what might cause differences in weevil response to different nematode species we also examined the behaviour of both nematode species on the body of the insect host.

METHODS

Nematodes

The nematodes, *S. carpocapsae* All and *H. downesi* K122, were cultured with late-instar larvae of the wax moth *Galleria mellonella* (Woodring & Kaya 1988). Infective juveniles were stored in tap water (2000 infective juveniles/ml) at 9 °C and were used within 3–5 weeks after emergence from the host cadaver. All experiments were repeated using different batches of infective juveniles.

Insects

Pine weevils develop in timber of recently killed conifers, such as stumps of felled trees. Adults emerge and feed on the bark and cambium of young trees. Although the adults feed above ground, they also spend much of their time resting or hiding at the soil surface (Nordlander 1987) where they would be exposed to edaphic pathogens including entomopathogenic nematodes. Adult pine weevils were collected from clearfelled coniferous forests in Ireland. They were stored for up to 4 weeks in ventilated boxes at 20 °C and fed fresh conifer billets (short lengths of stem). Pine weevil adults live for several years (Leather et al. 1999) and survive well in the laboratory. Within an experiment, weevils were assigned to different treatments randomly, irrespective of sex.

Grooming Response

We applied 1000 infective juveniles of either *S. carpocapsae* or *H. downesi* in 5 μl of water to a weevil's head region at the point closest to the top of the rostrum. Control weevils had 5 μl of water applied to the same area. The insect was then placed in a petri dish, 15 cm in diameter, and observed continuously for 20 min. We assigned the insect's behaviour to five mutually exclusive classes and recorded the number of incidences and the total length of time spent in each class, using the Observer software (Noldus Information Technology, Wageningen, The Netherlands). The behavioural classes were: (1) grooming head: the insect rubbed its head region with one or more of its legs; (2) grooming antennae: the insect rubbed its antennae with one or more of its legs; (3) grooming elytra: the insect rubbed its wing cases with one or more of its legs; (4) grooming legs: the insect rubbed one leg with another leg; (5) not grooming: the insect did not rub one part of its body against another.

Each insect (and nematode) was used once only. The experiment was run three times with 10 or 20 (depending on availability; total 50) insects per treatment. For each replicate we used a different culture batch of nematodes because culture batches of entomopathogenic nematodes may vary in behaviour and other attributes, even when cultured and stored using consistent protocols.

Behaviour of Nematodes on Weevils

We applied nematodes to weevils as above to investigate the behaviour of the nematodes on the weevils, although the number of nematodes was reduced to facilitate accurate recording. To prevent grooming, we restrained the weevil from moving its legs by wrapping its abdomen and thorax in several layers of laboratory film (Parafilm). We applied 100 infective juveniles of either