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Distribution of entomopathogenic nematodes in an Irish sand dune system

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Summary – A 100 × 800 m section of the sand dune system at North Bull Island, Dublin Bay, Ireland, was surveyed for entomopathogenic nematodes (EPN) by baiting soil cores with *Galleria mellonella* in July and August of 2001 and 2002. Two species were found: *Steinernema feltiae* (2.5 and 3.2% of cores) and *Heterorhabditis downesi* (1.9 and 3.8% of cores in 2001 and 2002, respectively). In each year, two colour variants of the *G. mellonella* cadavers containing *H. downesi* were found: purple and yellow. In both 2001 and 2002, *H. downesi* was most prevalent in the front 20 m of the dune system. *Steinernema feltiae* occurrence generally increased with distance into the dunes. In 2002, *H. downesi* was recovered most often in sandy paths than any other habitat surveyed but there was no habitat differentiation for *S. feltiae.* Human traffic may be an important factor in the distribution of both EPN and their insect hosts on Bull Island.

Keywords – coastal, dioecious, hermaphrodite, *Heterorhabditis*, parasite, *Photorhabdus*, predator, sex, soil, *Steinernema*, survey, *Xenorhabdus*.

Entomopathogenic nematodes (EPN) of the genera *Steinernema* and *Heterorhabditis* (Nematoda: Rhabditida) are obligate parasites of insects and some species are traded globally as biological control agents. Infective juvenile (IJ) nematodes emerge into the soil from natal insect cadavers before searching for new hosts. Having entered the host insect haemocoel, the IJ release symbiotic bacteria that kill the host after 24-48 h through septicaemia. *Steinernema* spp. are almost universally dioecious – IJ develop into amphimictic adults. In the case of *Heterorhabditis* spp., however, separate sexes are never produced in the first generation maturing in a new host, as each invading infective juvenile develops into a hermaphroditic female. Following development in liquid culture, the second generation of *Heterorhabditis megidis* (strain HSH 1) consist of females (30%), males (38%) and hermaphrodites (32%) (Strauch *et al.*, 1994).

Populations of EPN are spatially patchy (*e.g.*, Stuart & Gaugler, 1994, Spiridonov & Voronov, 1995) and prevalence is often low (Griffin *et al.*, 1991, 1994; Stuart & Gaugler, 1994; Campbell *et al.*, 1995; Hominick *et al.*, 1996). *Heterorhabditis* are frequently isolated from coastal areas (*e.g.*, Griffin *et al.*, 1991, 1994, 1999, 2000; Poinar, 1993; Liu & Berry, 1995) but their distribution is likely to be species-specific as *H. bacteriophora*, for example, occurs in silt loam soil rather than open textured soil (Hominick, 2002). *Steinernema* spp. have also been isolated from coastal regions (Amarasinghe *et al.*, 1994; Choo *et al.*, 1995; Griffin *et al.*, 2000) but appear to be more frequently isolated inland (Hominick & Briscoe, 1990; Griffin *et al.*, 1991; Hara *et al.*, 1991; Boag *et al.*, 1992; Choo *et al.*, 1995; Liu & Berry, 1995; Gwynn & Richardson, 1996; Hominick *et al.*, 1996, Hominick, 2002). Again, *Steinernema* distributions may be species-specific, or may be a function of sampling effort (Hominick, 2002).

The comprehensive survey of the Irish Republic by Griffin *et al*. (1991) revealed three species of EPN: *Steinernema feltiae* was more prevalent than *S. affine* (7.1 and 3.3% of samples, respectively), while *Heterorhabditis* was isolated from only one sample. A subsequent survey of Ireland and Britain by Griffin *et al.* (1994) sampled only sandy locations and resulted in *Heterorhabditis* being detected at 18/169 sites, with all positive sites being coastal. The *Heterorhabditis* isolates of these two studies were identified as belonging to the Irish group

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of *Heterorhabditis* and were subsequently known as *Heterorhabditis* 'Irish Type', until they were formally named *Heterorhabditis downesi* (Stock *et al.*, 2002). The restriction of *H. downesi* to coastal sandy soils only in Ireland, would explain the absence of the species in a survey of forest and field soils in Northern Ireland and the Republic of Ireland by Dillon *et al.* (2001) where only *S. feltiae* and *S. affine* were recovered. *Heterorhabditis downesi* has also been isolated in Hungary from pasture and roadside verge soils (Griffin *et al.*, 1999).

North Bull Island lies 7 km north east of Dublin City centre, off the coast of Raheny, in Dublin Bay, Ireland (longitude 6°6′ west, latitude 51°20′ north: National Grid Reference, O 2438). The island is a consolidated sandbar covering approximately 290 ha and consists of three main habitats: saltmarsh (70 ha), dune grassland (75 ha) and golf-links (modified dune grassland: 93 ha) (Wolfe & Hayden, 1996). As well as being a National Nature Reserve and a Dublin City park, North Bull Island was made a UNESCO Biosphere Reserve in 1981. The island is linked to the mainland by both a road bridge and a causeway, which greatly facilitate visitor access for the 1 000 000 inhabitants of Dublin City.

Moore (1977) gives a detailed account of the vegetation of the Bull Island dune complex. There are no higher plants growing between high neap and high spring tides. Annual plants such as saltwort (*Salsola kali*), sea rocket (*Cakile maritime*) and *Atriplex* species form embryonic dunes on accumulations of sand around seaweed deposited by equinoctial spring tides. The sand couchgrass (*Elymus farctus*) is common on the front first dune ridge, before both lyme grass (*Leymus arenarius*) and marram grass (*Ammophila arrenaria*) become more prevalent at the top of the first dune ridge. Past the second dune ridge, mosses prevent sand erosion by the wind and leguminous plants such as bird's foot trefoil (*Lotus corniculatus*) are present. A deeper layer of humus-rich soil after the second dune ridge enables more annual higher plants to colonise the area, creating a more stable grassland environment. The flora of Bull Island, which represent approximately 30% of the flora of Ireland, are presented by Forde (1999).

As mentioned above, *H. downesi* and *S. feltiae* have both been isolated from coastal habitats, but their coexistence on a fine scale and whether they share exactly the same habitat have yet to be investigated. Here, we expand on previous sampling of Bull Island that showed the presence of both *H. downesi* and *S. feltiae* in the dune system of North Bull Island (Downes, unpubl.) and examine the fine-scale distributions of the two species.

Materials and methods

The spatial distribution(s) of entomopathogenic nematodes in the sand-dune system of Bull Island was examined over a 2-week period from the end of July to the beginning of August in both 2001 and 2002.

Sampling method

Soil cores (10×10×150 mm) were taken from precisely the same sampling points in each year. The points were at 10 m intervals in a grid 100 × 800 m. The long axis of the grid paralleled the long axis of Bull Island (on a bearing of 60° east), with the short axis running perpendicular to this, into the sand dunes, on a bearing of 330° north. The most seaward core for each perpendicular bearing was taken below the high water spring tide mark.

Soil baiting

Each soil core was placed into a separate 25 ml universal specimen tube (Plastiques Gosselin, Borre, France). The core was not deliberately mixed but for cores with higher sand content some mixing was unavoidable. One final instar larva of the wax moth *Galleria mellonella* was placed on top of the soil (Bedding & Akhurst, 1975) and the universal tube was inverted and incubated for 5 days at 20°C. Bait insects were then removed, washed and placed individually into 5 cm Petri dishes lined with 5.5 cm filter paper (Whatman 1) moistened with water. They were stored at 20°C for a further 5 days whilst the soil samples were baited a second time, placing the new insect at the site from which the previous insect was removed. This meant an insect was present at the same location in each tube for a total of 10 days. After storage, any dead larva exhibiting the properties of an EPN kill was placed on a White (1927) trap to collect any emerging IJ. These emerged IJ were exposed to fresh *G. mellonella* larvae, and, 5 days after larval death, cadavers were dissected in quarter-strength Ringer's solution (Oxoid, Basingstoke, UK). Adult nematodes were identified morphologically. Cadavers that did not produce emergence were also dissected and any adult nematodes identified as previous.

Habitat survey

The habitat immediately surrounding the position from which each of the 880 soil cores were taken in 2002 was recorded as one of the following six categories: Pre-Dune/Beach, Marram Grass, Closed Grassland, Sandy Path, Grassy Path and Other.

Results

The only species of entomopathogenic nematode recovered from Bull Island in both 2001 and 2002 were *Heterorhabditis downesi* and *Steinernema feltiae*.

Overall prevalence

In 2001, from a total of 880 cores, 39 yielded EPN: an overall prevalence of 4.4%. This increased to 6.9% (61/880 cores) in 2002. The difference between the 2 years was significant (Two Proportion Test: $z = 2.13$, $P < 0.05$). *Heterorhabditis downesi* was significantly more prevalent in 2002 than in 2001 ($\chi^2 = 5.27$, $P < 0.05$) but there was no difference between years for *S. feltiae* ($\chi^2 = 0.741$, $P > 0.05$). Each species had a low prevalence of 2.8% (50/1760 cores) for both 2001 and 2002 combined. In 2001, second bait insects only were invaded by *H. downesi* on two occasions but this did not occur in 2002. *Steinernema feltiae* invaded second bait insects on eight occasions in 2001 and on three occasions in 2002. For *S. feltiae*, in 2001, only one soil core yielded EPN for both bait insects – a solitary male invaded the first bait, whilst a solitary female invaded the second bait insect. In 2002, no cores resulted in both first and second bait infections by *S. feltiae*.

In 2001, five of the 25 *G. mellonella* cadavers that were positive for *S. feltiae* yielded emerging IJ. This proportion was doubled (11/28) in 2002. In each year, each host cadaver not exhibiting emergence contained only one adult nematode, and there was no significant difference between the number of males and females found in these cadavers (One Proportion Tests, $P > 0.05$). Emergence of *S. feltiae* IJ indicates the presence of at least one male and one female in the cadaver. Because total numbers were inconveniently high for the calculation of Fisher's Exact Test, all entries were divided by four. Resulting entries were rounded down, in the case of cadavers that resulted in emergence, and rounded up in the case of infected insects that produced no F1 generation. Even with this conservative procedure, Fisher's Exact Test (two-tailed) indicated that in each year, emergence occurred significantly more often than would be expected if males and females are equally frequent, randomly distributed and found one another by chance (Fisher's Exact Test, $P = 0.0337$ for 2001; $P = 0.0006$ for 2002).

In each year, *H. downesi* prevalence peaked at 20 m into the dunes before declining to a lower, approximately stable level from 40-100 m (Fig. 1). In 2001, *S. feltiae* became more prevalent with distance into the dunes.

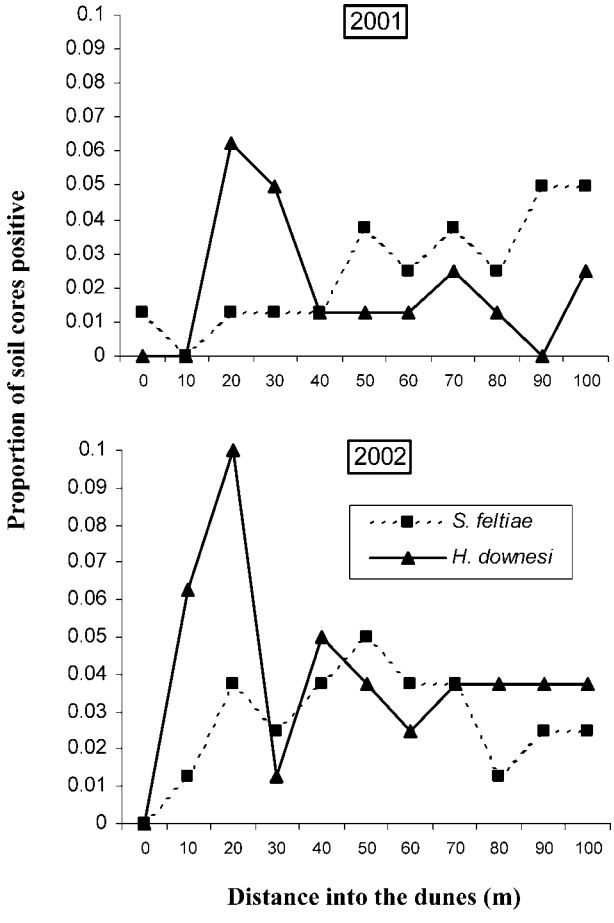

Fig. 1. *The prevalence of two species of entomopathogenic nematode in 2001 and 2002 as distance increases from the beach (0 m) into the sand dunes (up to 100 m) over a sampling area of 80 000 m^2 of Bull Island.*

By contrast, in 2002, after 50 m, *S. feltiae* prevalence appeared to decrease with distance into the dunes.

Colour phenotypes of *H. downesi* cadavers

Galleria mellonella larvae infected with *H. downesi* showed two phenotypic variants: a cadaver that changed colour from an initial pink to a golden yellow between 4 and 10 days after infection, and a cadaver that changed from an initial grey colour to a purple-brown after a similar time period.

On no occasion did either colour phenotype have significantly greater prevalence over the other phenotype in the front or rear 50 m of the dunes (Two Proportion Tests: $z = -0.27$, $P > 0.05$; $z = 1.81$, $P > 0.05$ for 2001 and 2002, respectively). This is despite the apparent dominance of the purple phenotype over the

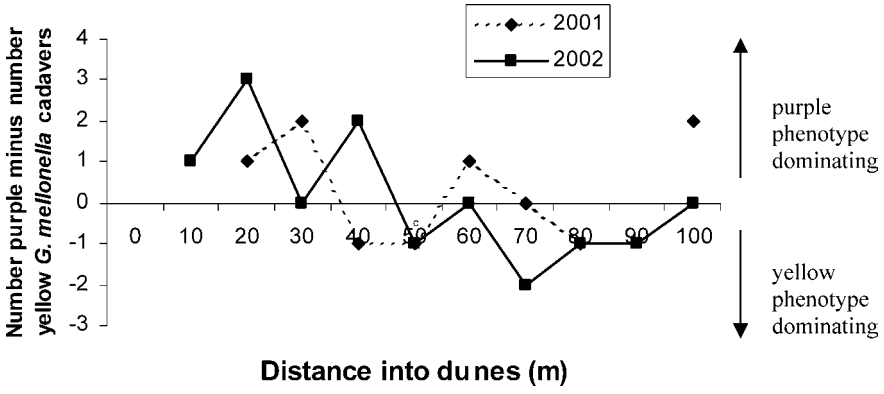

Fig. 2. *The relative dominance of purple and yellow phenotypes of* Heterorhabditis downesi *with increasing distance from the beach in 2001 and 2002.*

Table 1. *Distribution among habitats, and EPN yields of soil cores taken from an 80 000 m^2 area of sand dune system at Bull Island in July and August 2002.*

Habitat	Number (and proportion) of soil cores taken	Number (and %) of soil cores yielding EPN
Predune/Beach	108 (12.27%)	3 (4.92)
Marram Grass	236 (26.82%)	13 (21.31)
Closed Grassland	234 (26.59%)	15 (24.59)
Sandy Path	194 (22.16%)	23 (37.7)*
Grassland Path	102 (11.59%)	7 (11.48)
Other	5 (0.57%)	0 (0)
Total	880 (100%)	61 (100)

* Excluding 'Other' data, chi-square test gave a probability value close to significance ($\chi^2 = 9.36$, $P = 0.0526$). Removal of Sandy Path data from this analysis resulted in no significant difference between the remaining habitats ($\chi^2 = 2.03$, $P = 0.566$).

yellow phenotype in the front 50 m in 2002 (Fig. 2). Neither phenotype was significantly more prevalent in one half of the dunes over the other in either year (Two Proportion Tests, $-1.07 < z < 1.81$, $P > 0.05$).

Soil sample habitat survey

The two habitats most commonly found at core sites were Marram Grass and Closed Grassland (Table 1). By inspection, the percentage of cores positive for both *H. downesi* and *S. feltiae* combined was lower than expected (based on representation in the sampled population) for the Predune/Beach habitat, and higher than expected for the Sandy Path habitat. Chi-square applied to this data (excluding 'Other' data) gave a probability value close to significance ($\chi^2 = 9.36$, $P = 0.0526$). Removal

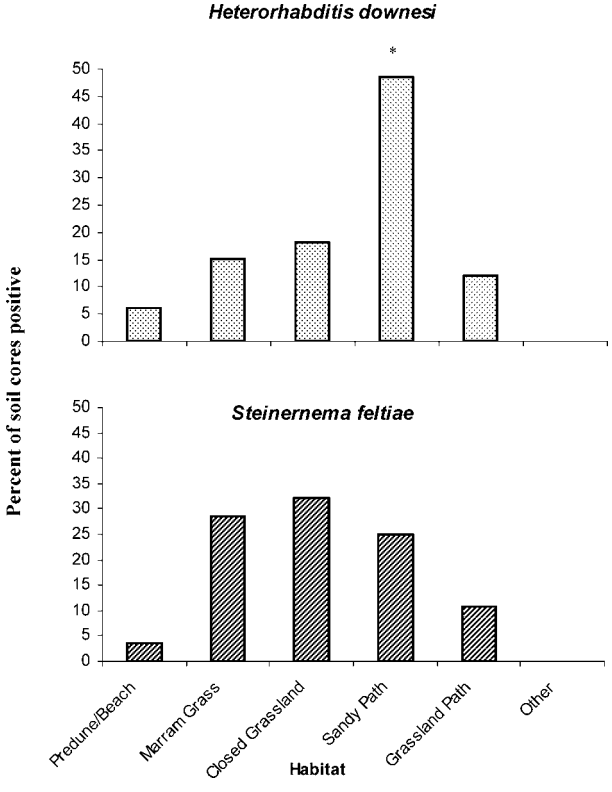

Fig. 3. *Proportion of 2002 Bull Island soil cores from different habitats that yielded either* Heterorhabditis downesi *or* Steinernema feltiae. *Asterisk indicates significant difference between habitats ($\chi^2 = 13.76$,* P *= 0.0032). Removal of Sandy Path data from this analysis resulted in no significant difference between the remaining habitats.*

of the Sandy Path data from this analysis resulted in no significant difference between the remaining habitats. For the purpose of analysis, the Predune and Grassland Path data of Figure 3 were combined for each species sepa-

rately to make the data suitable. Chi-square applied to the resulting 2×4 tables revealed no significance between habitats for *S. feltiae*. *Heterorhabditis downesi* showed significant difference between habitats ($\chi^2 = 13.76$, $P = 0.0032$). The removal of the Sandy Path data for this analysis showed that there was no significant difference between the remaining habitats for *H. downesi*.

Discussion

The survey of Ireland and Britain by Griffin *et al.* (1994), specifically for *Heterorhabditis* spp., concentrated on sandy locations. Importantly, all of the positive sites were coastal with no recoveries from 40 inland sites. *Heterorhabditis downesi* and *S. feltiae* have previously been frequently recovered from the same coastal sandy grassland habitats in Ireland (Griffin & Downes, unpubl.). The prevalence of both *H. downesi* and *S. feltiae* in Bull Island is low (2.5%) compared to the findings of Griffin *et al.* (1991, 1994) but of the various soil textures sampled by Griffin *et al.* (1991), *S. feltiae* was found only once in sandy soil, with the majority of *S. feltiae* being recovered from loamy soil. The volume of soil per Bull Island core (15 cm^3) is notably less than that taken by Griffin *et al.* (1991) (approximately 0.4-0.5 kg per sample, each consisting of approximately 40 bulked sub-samples), and may also help to explain the difference in number of cores/samples positive for *S. feltiae* and *H. downesi*. Importantly, Griffin *et al.* (1991) focused on incidence of EPN (sites where EPN were present) whereas this study was concerned with EPN intensity (the population level of EPN at a single site).

When no *S. feltiae* F1 generation was produced from infected insects, extraordinarily, on no occasion did more than one nematode of the same sex invade the same bait insect. This, taken along with the unexpectedly high occurrence of emergence (meaning male and female occurring together), or alternatively the lower than expected numbers of lone invaders, raises questions regarding the distribution and invasive behaviour of same sex individuals under natural conditions.

The natural hosts of *S. feltiae* and *H. downesi* on Bull Island are unknown, so it is only possible to speculate whether the different distributions of the two EPN species within the dune system are due to differing host preferences and host distributions. The black marram weevil, *Otiorhynchus atroapterus*, is frequently observed throughout the dune system at Bull Island (Rolston, pers. obs.) and its larvae may be an important host for EPN. The larvae of lepidoptera, diptera and other beetles, such as the ground-dwelling *Laemostenus terricola*, may also act as host insects.

The life history strategy of hermaphroditism adopted by *H. downesi* may be more successful than the separate sexes of *S. feltiae* in the heterogenous environment of the foredunes and may help to explain the dominance of *H. downesi* in this habitat. Sex may be a strategy for EPN not only to cope with selection pressures placed on them by their sexual hosts, but also for EPN to cope with their own parasites and predators by introducing genetic variation within their progeny (see Hamilton *et al.* (1990) for a review on the value of sex). Predators and parasites of EPN are likely to be more prevalent in the more stable rear dunes, raising a requirement for separate sexes in this area of the dunes. *H. downesi* may be able to exist in all areas of the Bull Island dune system because: *i*) hermaphroditism at the first generation allows it to have low characteristic densities despite the reduced chance of finding a mate in the unstable foredunes; *ii*) the low characteristic densities may not encourage abundance of its parasites; and *iii*) being capable of sexual reproduction in the second generation may allow *H. downesi* enough genetic variation to cope with the pressures exerted in the more stable rear dunes. *Steinernema feltiae* may have lower prevalence in the foredunes because it is unable to exist at such low characteristic densities as *H. downesi*.

Griffin *et al.* (1994) noted the occurrence of different *H. downesi* colour phenotypes at different sites in Ireland and Britain, but this is the first report of two colour phenotypes occurring within the same site. The two commonest phenotypes of *H. downesi* cadavers noted by Griffin *et al.* (1994) were those found on Bull Island: yellow and purple/green. The *Photorhabdus* symbiont isolated from these nematodes are yellow and achromatic, respectively, when grown *in vitro*. The biological significance of these differently pigmented symbionts is not known. *Heterorhabditis downesi* is not the only EPN to produce between-isolate cadaver colour variation. Green and red phenotypes of *H. bacteriophora* have also been reported from different sites (Tarasco *et al.*, 2003). Other isolates of *H. downesi* also exhibiting these cadaver colours include the two isolates on which the description of *H. downesi* is based: EU349 from Hungary (purple/green), and K122 from Ireland (yellow) (Griffin *et al.*, 1999). Szallas *et al.* (2001) found that K122 and EU349 carry symbionts that differ in their 16S rDNA sequences, and also in their IGS PhastSystem PCR-RFLP patterns. Although K122 and EU349 are indistinguishable morphologically

(Stock *et al.*, 2002) and by RFLP patterns (Pamjav *et al.*, 1999), they are each unable to utilise the others' symbiotic bacteria (Boszormenyi *et al.*, 2001). Perhaps the isolates carrying the yellow and purple bacteria on Bull Island are similarly restricted.

Only on one occasion (in 2001) was *S. feltiae* found below the High Water Spring Tide (HWST) strandline. In 2002, *H. downesi* was found at approximately the HWST mark on three occasions. *Heterorhabditis* has previously been recovered from unvegetated shore in Sri Lanka (Amarasinghe *et al.*, 1994). This habitat is subject to high salinity and desiccation and seems not to be a favourable habitat for EPN. It is possible that these positive sites are a result of movements of sand by wind and/or animals and humans. However, abundant insects (mainly Coleoptera and Diptera) may be found feeding amongst strandline detritus (Williams, 1980), and could represent large populations of potential EPN hosts, as suggested by Hominick *et al.* (1996). Both *Steinernema* spp. and *Heterorhabditis* spp. are capable of infecting and reproducing within terrestrial isopods (Poinar & Paff, 1985; Poinar, 1989). Amphipods such as *Talitrus saltator* inhabit non-permanent burrows just above the high water strandline, and are frequently found well above the extreme HWST mark (Bregazzi & Naylor, 1972). These burrows might bring such amphipods into contact with EPN. Moulting *T. saltator*, which would be expected to be more susceptible to EPN infection, are distributed mainly in the upper regions of the burrowing zone (Williams, 1978, 1980). Thus EPN found in the predune or beach habitats may be exploiting this rich source of hosts.

Throughout Bull Island, human activity has created pathways through the vegetation of the dune system. On the more frequently used paths the vegetation is completely absent, revealing bare sand. The habitat from which *H. downesi* was most frequently isolated was these Sandy Paths. Sand dunes represent an unfavourable environment for invertebrates due to little vegetation cover at the ground surface and low organic matter in the dune soil that does not hold water well. Although sand warms quickly, it cools rapidly or dries out (Speight, 1977). Therefore, low vegetation cover of the sandy paths would lead to the expectation of few insects and therefore few EPN. The high presence of *H. downesi* in paths may reflect its disappearance in vegetated areas, either into insects or because of attack by predators and pathogens. In addition, high levels of human traffic on the sandy paths of Bull Island may be responsible for some of the EPN occurrence there. However, nematodes transferred by humans are likely to be deposited on the surface of the path, an environment subject to high desiccation, low moisture levels and high UV exposure. Perhaps elevated numbers of herbivorous insects associated with plant damage and insect transfer along the paths are important (Mráček & Webster, 1993). If below-ground herbivores are favoured by trampling in this habitat then the effects of human impact on the distribution of pest insects and therefore of EPN may be greatly underestimated.

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