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Steinernema feltiae: Ammonia triggers the emergence of their infective juveniles

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Abstract

Entomopathogenic nematodes complete their life cycles inside dead insects. The emergence of new infective juveniles from the cadaver has been attributed (but never demonstrated) to food depletion or to the accumulation of metabolites from the breakdown of the host's tissues. Here we give evidence that emergence is triggered by ammonia, a product of nematode defecation. We found that the emergence of *Steinernema feltiae* infective juveniles from *Galleria mellonella* cadavers was stimulated by a particular level of ammonia. Emergence was delayed when ammonia in the cadaver was decreased and was prompted when increased. These findings will further improve the understanding of the nematode life cycle. Here we speculate that production of infective juveniles can be mediated by ammonia and work in a manner analogous to that of the dauer recovery inhibiting factor (DRIF) in *Caenorhabditis elegans*.

Index Descriptors and Abbreviations: Ammonia; Defecation; *Steinernema feltiae*; *Caenorhabditis elegans*; *Caenorhabditis briggsae*; *Panagrellus redivivus*; *Ascaridia gallis*; *Heterorhabditis bacteriophora*; *Xenorhabdus bovienii*; *Galleria mellonella*; Behaviour; Dauer; Food depletion; Life cycle; Entomopathogenic nematodes (EPN); Infective juvenile (IJ); Dauer recovery inhibiting factor (DRIF); Ammonia triggering point (ATP)

1. Introduction

Nematodes generally are considered as aquatic organisms and their most efficient way to excrete nitrogen-waste compounds is in the form of ammonia, which is highly toxic but which does not represent a problem in aquatic environments because it is rapidly dissolved in water. In terrestrial ecosystems, the nematodes need water to survive and they do so by living within thin films of water in the soil aggregates and pores. Ammonia constitutes about 40–90% of non-protein nitrogenous excretions of nematodes, and in some species urea could also be important (Wright, 1998). Indeed, the characterization of the excretion products of different nematodes such *Caenorhabditis briggsae* (Rothstein, 1963), *Panagrellus redivivus* (Wright, 1975) or *Ascaridia gallis* (Rogers, 1952) has shown large quantities of ammonia.

Entomopathogenic nematodes (EPN) are soil dwelling organisms that in order to survive and complete their life cycle, must parasitize and kill an insect host, they can also survive by scavenging insect bodies (San-Blas and Gowen, 2008). The insect's death is the result of an infection produced by a symbiotic bacterium which is located in the digestive tract of the nematode, and which is released when the nematode penetrates into the insect's body.

The life cycle of EPN is completed inside a host and depends on the availability of food resources. Two or three generations can be produced. After the infective juvenile (IJ) has entered into the host, it moults to fourth juvenile stage (J4) and eventually becomes adult. The adults lay eggs and after hatching the new juveniles J1 develop through J2, J3, and J4, repeating the process again. But at a certain point, J2, instead of moulting to J3, start changing to the non-feeding and dispersal stage IJ (it is

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believed that this occurs due to the food depletion). These nematodes abandon the host cadaver searching for another host to invade and so complete their life cycles.

Most authors agree that the emergence of IJ is triggered by either overcrowding or food resource deficit (Nguyen and Smart, 1992; Ryder and Griffin, 2002). Similarly, in the rhabditid *Caenorhabditis elegans* the development of the non-feeding dispersal stage has been linked to high population densities and limited food supply (Cassada and Russell, 1975). O'Leary et al. (1998) mention that the emergence of IJ could be mediated by the nutrient status of the insect cadaver, by the accumulation of metabolites from the breakdown of the host's tissues or from the metabolism of the symbiotic bacteria.

Ammonia is known to have nematicidal effects on some plant parasitic species (Rodriguez-Kabana, 1986; Oka and Pivonia, 2004; Oka et al., 2006); on the other hand, Pye and Burman (1981) reported random dispersion of *Steinernema carpocapsae* in agar plates with a NH_4^+ gradient. Shapiro et al. (2000) mentioned that infective juveniles of *Heterorhabditis bacteriophora* were attracted to an ammonia concentration of 0.4% of NH_4OH but were repelled by concentrations of NH_4OH above 4%. Many EPN species have shown avoidance behaviour when high amounts of ammonia were present in insect faeces (Grewal et al., 1993).

Wright (2004) postulated that high concentrations of ammonia can occur when a nematode population is severely overcrowded or where the diffusion of this compound is difficult; therefore it is possible that after a few days an insect infected by EPN can fulfil at least the first condition, and probably the second depending on the moisture level of the surrounding environment.

The reasons governing, or the mechanisms for the emergence of EPN from the insect host have been poorly understood since the primary assumption was made that it could be due to food depletion. But this does not explain how or why the infective juveniles can recognize this lack of food as a signal for emerging from the cadaver. Moreover, when the emergence starts, the insect cadavers may still contain material (broth of insect tissue and bacteria). There is an ecological reason which remains unclear. The host cadaver itself represents probably the best refuge for the nematodes because the bacterial content inhibits secondary infection (Boemare et al., 1992), possibly protecting the nematodes against pathogens, as well as against harsh environmental conditions such as frost (Lewis and Shapiro-Ilan, 2002) or drought (Koppenhöfer et al., 1997; Pérez et al., 2003). Therefore the reasons for the nematodes to abandon the cadaver and undertake the risk of searching for a new host could be either related to genetic factors or might be a response from a single or a group of chemical stimuli, or even both.

The objective of this paper was to examine if the concentration of ammonia (the product of excretion) in an insect infected by EPN can be the chemical trigger responsible for emergence of the infective juveniles.

2. Materials and methods

2.1. Nematode culture

The species *Steinernema feltiae* (Rahbditida: Steinernematidae) was cultured in the fourth instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae) (Livefoods Direct Ltd., Sheffield, UK) following the Dutky et al. (1964) technique. The infected larvae were incubated at 20 °C. The infective juveniles were collected using adapted White traps (White, 1927) and were stored at 10 °C until the experimentation day (no more than 2 weeks).

2.2. Bacteria source

The symbiotic bacteria *Xenorhabdus bovienii* (Enterobacteriales: Enterobacteriaceae) used for the experiments were taken from *G. mellonella* larvae which had been exposed to *S. feltiae* 48 h before. The infected *G. mellonella* cadavers were surface sterilized with 70% methanol for 10 min, after which they were dissected under sterile conditions and the body content was used as the bacterial source. Five samples of the body content of each *G. mellonella* used were plated on selective medium of nutrient bromothymol agar (NBTA) (3.3% nutrient agar, 0.0025% bromothymol blue and 0.004% 2,3,5-triphenyltetrazolium chloride). The plates were incubated for 48 h at 20 °C to confirm *Xenorhabdus* spp. presence (colonies grow dark blue in NBTA medium).

2.3. Determination of ammonia in G. mellonella larvae

Four hundred and fifty *G. mellonella* larvae (fourth instar) were placed individually in 96-well plates ∅2 cm filled with silver sand (BDH Laboratory Supplies, Poole, UK). Three treatments were prepared: 150 larvae were exposed to 20 *S. feltiae* IJ per well; 150 were inoculated with *X. bovienii* by puncturing the body of the *G. mellonella* larvae with a needle containing the bacteria from the body content previously mentioned (this was repeated once in every *G. mellonella*), and the control treatment was with 150 *G. mellonella* which were killed previously by freezing and placed in the plate wells. The plates were kept in an incubator at 20 °C during the time of the experiment. Immediately after starting the experiment (0 h) and every 24 h, 10 *G. mellonella* from each treatment were taken randomly and the ammonia content was measured for 14 days (total 336 h).

The quantity of ammonia was determined modifying the Dorich and Nelson (1983) technique for direct colorimetric measurement of ammonium in 2 M potassium chloride extracts from soil samples. The modifications of the technique were: (1) The body contents of the *G. mellonella* larvae were exposed by dissection and were weighed; 2 M KCl was added at a rate of 5 ml 2 M KCl g of *G. mellonella*$^{-1}$. (2) After incubation, 0.5 ml of the resulting suspension was extracted and centrifuged at 6000 rpm for 10 min. (3) An