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# Does osmoregulatory behaviour in entomopathogenic nematodes predispose desiccation tolerance?

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**Summary** – Infective juveniles of four isolates of entomopathogenic nematodes placed into balanced (mixed) salt solutions displayed varying levels of ability to osmoregulate under hyperosmotic conditions. The isolate with the greatest desiccation tolerance (*Steinernema carpocapsae* SCB-L1) showed the greatest osmoregulatory ability. However, the isolate with the poorest desiccation tolerance (*Heterorhabditis* sp. HIS-33) could also osmoregulate. One isolate with moderate desiccation tolerance (*H. bacteriophora* HP88) had the poorest osmoregulatory ability while another (HIS-28) showed good osmoregulatory ability. This shows that there is not necessarily a close link between hypo-osmotic regulation and desiccation tolerance. The need for separate screens for these different survival attributes is, therefore, indicated. Assessment of *Heterorhabditis* sp. isolate HIS-28 found that osmoregulatory ability and percent survival was greater in balanced salt solutions compared with single salt solutions, providing indirect evidence for a specific ionic regulation mechanism.

**Keywords** – *Heterorhabditis*, *Steinernema*, survival.

Infective juveniles (IJ) of steinernematid and heterorhabditid entomopathogenic nematodes are the free-living survival stage and are often exposed to varying environmental conditions (Bednarek & Gaugler, 1997). Previous work has shown that steinernematids and heterorhabditids can show osmoregulatory ability in hyper-osmotic conditions (Piggott *et al.*, 2000). Osmoregulatory ability was shown to be variable according to the isolates assessed and it was suggested that this was related to their natural habitat.

Cuticle permeability is a key factor determining the ability of nematodes to survive evaporative or osmotic water loss but the specific sites involved are unknown (Wright, 1998). A comparison of wild type *Heterorhabditis megidis* with a mutant, whose greater tolerance to evaporative water loss has been related to a greater negative charge on its sheath (O'Leary *et al.*, 1998), failed to show a correlation with ability to regulate water content (Piggott *et al.*, 2000). However, it was not known if the mutant displayed a tolerance mechanism similar to that found naturally.

In the present study, hypo-osmotic regulation by natural isolates of entomopathogenic nematodes that vary in their desiccation tolerance were compared.

## Materials and methods

### NEMATODES

*Heterorhabditis bacteriophora* strain HP88 was obtained from Dr Randy Gaugler (Rutgers University, NJ, USA). Two other *Heterorhabditis* strains, designated HIS-28 and HIS-33, were isolated from coastal soils (at Kfar Hess and Hedera, respectively) in Israel (Glazer *et al.*, 1991; Liu & Glazer, 2000). A *Steinernema carpocapsae* isolate, designated SCB-L1, was obtained from Beijing, China by one of us (Liu). A *Steinernema* sp. (SSL85) isolate from Malaysia (Mason *et al.*, 1996) was used for preliminary studies to confirm the relationship between nematode volume and length. Nematodes were cultured in late instar larvae of the wax moth *Galleria mellonella* L. at 20°C (Woodring & Kaya, 1988). Infective juveniles

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emerging within the first 4 days were maintained in a balanced, isosmotic salt solution (25 mOsm $Kg^{-1}$; Piggott *et al.*, 2000).

### Experimental procedure

Distilled water suspensions (10 ml) of IJ from the different nematode strains, containing about 40 000 IJ, were concentrated onto a 5 cm diameter filter paper (Whatman No. 1) using a vacuum filtration apparatus (Liu & Glazer, 2000) and placed in glass desiccation chambers. Relative humidities (r.h.) of 97 and 85% were established in desiccation chambers with saturated salt solutions of $K_2SO_4$ and KCl, respectively, at 25 ± 0.3°C (Winston & Bates, 1960). Two desiccation regimes were used. The first exposed IJ to 97% r.h. for 72 h and the second to 72 h at 97% r.h. followed by a further 24 h at 85% r.h. Nematode survival was determined by cutting small sections from the filter paper discs, each with approximately 5000 IJ on its surface, and immersing them in distilled water for 24 h. Sub-samples (*ca* 100 IJ) were then taken for nematode survival determination. Nematode viability was determined by observing motility following a gentle prodding with a needle as described by Glazer (1992) under a stereomicroscope. Each treatment was replicated three times.

In the first osmoregulation experiment, sodium chloride was added to the balanced salt solution to adjust the osmolality to 500 and 1000 mOsm $kg^{-1}$ since balanced salt solutions >50 mOsm could not be used due to flocculation. Infective juveniles (*ca* 100 per treatment) of HP88, HIS-28, HIS-33 and SCB-L1 were placed into the test solutions and incubated for up to 48 h at 23-26°C. Controls were incubated in the maintenance solution (25 mOsm $kg^{-1}$). Survival of nematodes was recorded up to 48 h, based on assessment of movement of 100 IJ per treatment. All of the IJ examined in experiments were ensheathed.

Change in water content has been shown to vary proportionally with length in a number of nematode species (Wright, 1998). To determine the relationship between length and water content of ensheathed IJ, *Steinernema* sp. (SSL85) was incubated for varying periods in salt solutions (200-1000 mOsm $kg^{-1}$), with more than 500 IJ per treatment. At time 0, and after different incubation times, individual IJ (n = 20) were transferred to glass slides, their length (excluding sheath) measured using a graticule eyepiece under an inverted microscope and their water content estimated by quantitative interference microscopy. The same procedure was used to measure the length of IJ in the osmoregulation experiments. Each nematode was considered a replicate.

In a second osmoregulation experiment, balanced salt solutions supplemented with sodium chloride (see above) were compared with single salt, sodium chloride solutions of equivalent osmolality. *Steinernema* sp. (HIS-28) was used as a test organism and was incubated at 200, 500 and 1000 mOsm $kg^{-1}$ for up to 24 h at 23-26°C. Controls were incubated in the maintenance solution (25 mOsm $kg^{-1}$). Assessment of nematode length and survival was as described for the first experiment.

### Statistical analysis

For the nematode length assessment, a one-way ANOVA was used to determine significant changes in the water content of the nematode samples. The relationship between water content and length was analysed using linear regression. Survival data (%) from osmoregulatory experiments were logit transformed prior to ANOVA. Significant differences were determined at the 5% level; means were separated by the Tukey's multiple range test.

## Results

### Desiccation survival

Survival of nematode strains following desiccation for 72 h at 97% r.h. ranged from 88% for SCB-L1 to 74% for HIS-33 (Table 1). The differences between nematode strains were amplified when an additional period of desiccation (24 h) at 85% r.h. was used, giving three clear groupings for desiccation tolerance, where SCB-11 > HIS-28 / HIS-33 > HP88.

**Table 1.** *Desiccation survival of* Heterorhabditis *isolates HP88, HIS-28, HIS-33 and* Steinernema carpocapsae *SCB-L1 following desiccation at A: 97% r.h. for 72 h and B: 97% r.h. for 72 h followed by 24 h at 85% r.h. (n = 3).*

| Treatment | Mean survival[1] % (SE) | | | |
|---|---|---|---|---|
| | SCB-L1 | HIS-28 | HP88 | HIS-33 |
| A | 88 (4.7) | 82 (4.2) | 79 (4.2) | 74 (3.6) |
| B | 77 (6.7) | 34 (5.2) | 23 (4.7) | 5.1 (3.6) |

[1] 100% survival at time 0 h.

**Table 2.** *Mean length of* Heterorhabditis *sp. HIS-33 and HIS-28,* Steinernema carpocapsae *SCB-L1 and* Heterorhabditis bacteriophora *HP88 infective juveniles during incubation in balanced salt solutions supplemented with NaCl (n = 20).*

| Solution (mOsm kg$^{-1}$) | Time (h) | Mean length $\mu$m (SE)[1)] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | SCB-L1 | | HIS-28 | | HIS-33 | | HP88 | |
| 25[2)] | 0 | 556$^{a}$ | (3.4) | 617$^{a}$ | (2.3) | 608$^{a}$ | (2.8) | 615$^{a}$ | (2.6) |
| | 3 | 555$^{a}$ | (3.4) | 620$^{a}$ | (2.7) | 606$^{a}$ | (2.7) | 616$^{a}$ | (2.5) |
| | 6 | 550$^{a}$ | (3.1) | 619$^{a}$ | (2.6) | 610$^{a}$ | (2.8) | 617$^{a}$ | (2.8) |
| | 12 | 549$^{a}$ | (3.6) | 621$^{a}$ | (2.5) | 609$^{a}$ | (3.0) | 617$^{a}$ | (2.9) |
| | 24 | 556$^{a}$ | (3.9) | 620$^{a}$ | (2.5) | 609$^{a}$ | (2.6) | 615$^{a}$ | (2.4) |
| | 48 | 557$^{a}$ | (3.6) | 619$^{a}$ | (3.2) | 613$^{a}$ | (2.5) | 617$^{a}$ | (3.1) |
| 500 | 0 | 556$^{a}$ | (3.4) | 617$^{a}$ | (2.3) | 608$^{a}$ | (2.8) | 603$^{a}$ | (2.9) |
| | 3 | 532$^{b}$ | (3.8) | 594$^{b}$ | (2.8) | 557$^{b}$ | (2.8) | 591$^{b}$ | (4.4) |
| | 6 | 540$^{b}$ | (2.6) | 588$^{bc}$ | (2.7) | 551$^{b}$ | (2.9) | 579$^{c}$ | (3.8) |
| | 12 | 540$^{b}$ | (3.6) | 585$^{c}$ | (3.6) | 552$^{b}$ | (3.1) | 574$^{c}$ | (3.9) |
| | 24 | 542$^{b}$ | (2.9) | 608$^{d}$ | (2.8) | 595$^{c}$ | (3.1) | 571$^{c}$ | (3.8) |
| | 48 | 554$^{a}$ | (3.1) | 619$^{a}$ | (3.2) | 603$^{a}$ | (2.4) | 568$^{c}$ | (3.7) |
| 1000 | 0 | 556$^{a}$ | (3.4) | 617$^{a}$ | (2.3) | 608$^{a}$ | (2.8) | 603$^{a}$ | (2.9) |
| | 3 | 517$^{b}$ | (3.2) | 557$^{b}$ | (3.3) | 542$^{b}$ | (2.9) | 559$^{bc}$ | (4.2) |
| | 6 | 493$^{c}$ | (3.5) | 554$^{b}$ | (3.0) | 539$^{b}$ | (3.0) | 549$^{b}$ | (3.5) |
| | 12 | 536$^{d}$ | (3.5) | 550$^{b}$ | (3.8) | 527$^{c}$ | (3.1) | 554$^{bc}$ | (4.1) |
| | 24 | 544$^{d}$ | (3.4) | 591$^{c}$ | (3.6) | 566$^{d}$ | (3.6) | 561$^{c}$ | (3.0) |
| | 48 | 550$^{ad}$ | (3.1) | 615$^{a}$ | (3.5) | 596$^{e}$ | (3.1) | 557$^{bc}$ | (4.1) |

[1)] Values followed by a common letter are not significantly different ($P > 0.05$).
[2)] Control in which nematodes were maintained prior to the start of the experiment.

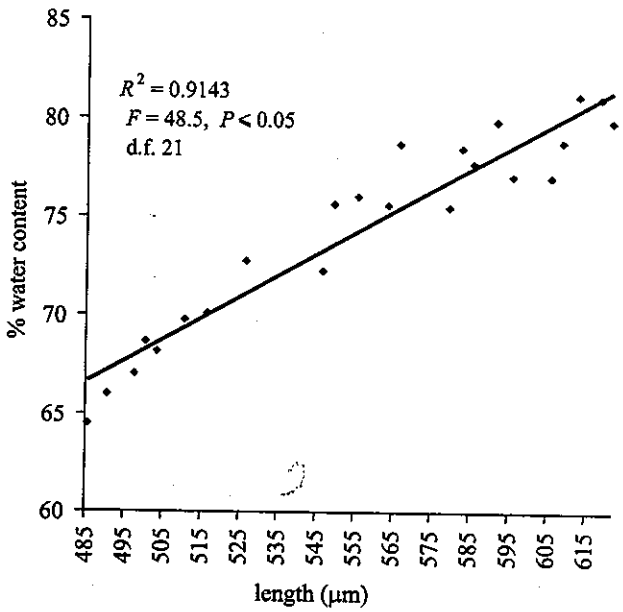


**Fig. 1.** *Relationship between water content and length of* Steinernema *sp. SSL 85 infective juveniles.*

### Relationship between body length and water content

*Steinernema* sp. SSL85 showed a significant positive linear relationship between body length and water content (Fig. 1; $P \leqslant 0.05$).

### Regulation of body length in balanced salt solutions

*Steinernema carpocapsae* SCB-L1 showed a maximum, 4% reduction in length ($P \leqslant 0.05$) after 3 h incubation at 500 mOsm kg$^{-1}$ and a full recovery in length ($P > 0.05$ compared with time 0) after 48 h (Table 2). At 1000 mOsm kg$^{-1}$, the maximum reduction in length (11%) occurred after 6 h ($P \leqslant 0.05$) with a full recovery ($P > 0.05$ compared with time 0) after 48 h. Both *Heterorhabditis* sp. isolates (HIS-28 and HIS-33) showed a significant ($P \leqslant 0.05$) reduction in length (5 and 9%, respectively) after 6-12 h incubation at 500 mOsm kg$^{-1}$ followed by a full recovery ($P > 0.05$ compared with time 0) after 48 h. For HIS-28 and HIS-33, the reduction in

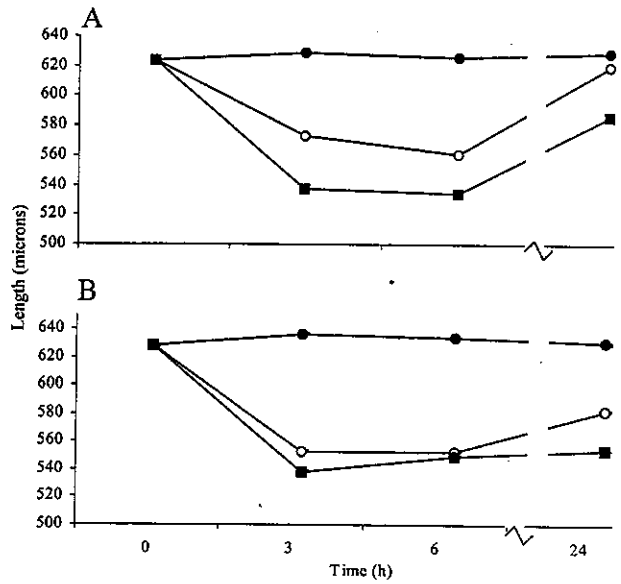


**Fig. 2.** *Change in length of infective juveniles of* Heterorhabditis *sp. HIS-28 during incubation in solutions of 25 (●), 500 (○) and 1000 (■) mOsm kg$^{-1}$. A: Balanced salt-solutions supplemented with NaCl; B: Single salt (NaCl) solutions.*

length was greatest after 12 h at 1000 mOsm kg$^{-1}$ (11 and 13%) with full recovery for HIS-28 ($P > 0.05$ compared with time 0) and a significant recovery ($P \leqslant 0.05$ compared with mean length measurements after 3-24 h) for HIS-33 after 48 h. *Heterorhabditis bacteriophora* HP88 showed a continual, and significant ($P \leqslant 0.05$), reduction in length over 3 to 48 h (to −6%) at 500 mOsm kg$^{-1}$. At 1000 mOsm kg$^{-1}$, HP88 showed a maximum reduction in length (9%) after 6 h with no significant ($P > 0.05$) recovery in length after 48 h. For the different treatments, survival of IJ after 48 h averaged 93-96% ($P > 0.05$).

### Regulation of *Heterorhabditis* sp. HIS-28 body length in balanced and single salt solutions

In the balanced salt solutions, HIS-28 showed a significant decrease ($P \leqslant 0.05$) in length (10 and 14%) after 6 h at 500 and 1000 mOsm kg$^{-1}$, respectively (Fig. 2). At 500 mOsm kg$^{-1}$, there was a full recovery in length after 24 h. At 1000 mOsm kg$^{-1}$, there was some ($P \leqslant 0.05$) recovery of length (to −6%) after 24 h. In single salt solutions, there was an initial (3-6 h) reduction in length of 12 and 14% ($P \leqslant 0.05$) at 500 and 1000 mOsm kg$^{-1}$, respectively. There was some recovery ($P \leqslant 0.05$) after 24 h in both cases (to −7 and −11%, respectively). Survival of IJ after 24 h in balanced salt solutions ranged from 95-100% ($P > 0.05$) for the different treatments. In the single salt solutions, survival of IJ averaged 97, 79 and 64% in the control (25), 500 and 1000 mOsm kg$^{-1}$ solutions after 24 h.

## Discussion

Based on the positive, linear relationship between nematode length and water content (Fig. 1; Wright, 1998), the present work confirms earlier observations (Piggott *et al.*, 2000) that the osmoregulatory ability of entomopathogenic nematode IJ in hyper-osmotic environments can vary markedly between species or isolates.

The present results also show that there is not necessarily a link between hypo-osmotic regulation, a process that involves combating water loss due to osmosis, and desiccation tolerance. The isolate with the greatest desiccation tolerance (*S. carpocapsae* SCB-L1) showed the greatest osmoregulatory ability in the balanced salt solutions with rapid control of water loss (after 3-6 h), particularly at 500 mOsm kg$^{-1}$, an osmolality equivalent to that of seawater. Similarly, *Heterorhabditis* sp. HIS-28, an isolate with moderate desiccation tolerance, showed good osmoregulatory ability. However, *Heterorhabditis* sp. HIS-33, the isolate with the poorest desiccation tolerance of those tested, also showed some osmoregulatory ability, while an isolate with moderate desiccation tolerance (*H. bacteriophora* HP88) showed the poorest osmoregulatory ability.

Desiccation tolerance in nematodes, including entomopathogenic species, is linked to mechanisms that slow the rate of water loss coupled with biochemical adaptations for the synthesis of polyols or other cell protectants (Womersley *et al.*, 1998; Solomon *et al.*, 1999). Likewise, osmoregulation in some nematodes has been shown to be due to both passive and active mechanisms (Wright, 1998). The possibility of a common mechanism for controlling water loss due to osmosis and water loss due to evaporation cannot, therefore, be ruled out. However, the lack of a consistent link between osmoregulation and desiccation tolerance observed in the present study suggests that, if such a common mechanism exists, it is not in itself sufficient for either process.

The need for separate screens of nematode isolates for these different survival attributes is, therefore, indicated. Nematode isolates that are particularly suited for survival and the maintenance of infectivity under hyper-osmotic conditions would be useful, for example, in saline soils or soils with high levels of amendments, and in formulations with high solute contents.

Assessment of *Heterorhabditis* sp. isolate HIS-28 found that osmoregulatory ability of IJ was greater in balanced salt solutions compared with single salt (NaCl) solutions. The percent survival of IJ was also reduced markedly in 500 and 1000 mOsm $kg^{-1}$ NaCl. These results indicate that the presence of one or more of the salts in the balanced solution is important for both osmoregulation and survival, providing indirect evidence for a specific ionic regulation mechanism.

Molecular studies are required to identify and characterise the mechanisms for water and ion regulation of entomopathogenic nematodes. *Caenorhabditis elegans* provides a heterologous system that can help determine the function of a wide range of genes and genetic pathways in other nematode species. For example, a putative $Na^+/K^+$ antiporter sequence has been reported in *C. elegans* (Marra *et al.*, 1993), together with cDNA for a water channel (Kuwahara *et al.*, 1997) and a gene (*flr-1*) encoding a novel $Na^+$ channel (Take-uchi *et al.*, 1998).

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