Effects of low storage temperature on survival and infectivity of two *Steinernema* species (Nematoda : Steinernematidae)

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SUMMARY

Survival of two British steinernematids, one a strain of *Steinernema feltiae* (Filipjev) [= *S. bibionis* (Bovien)], and the other an undescribed species designated *Steinernema* sp. (Nashes strain) was assessed after storage of infective juveniles in sterile sand at 5 °C and 15 °C and was measured by their motility (ability to be extracted by " mini " Whitehead trays). Infectivity after storage at the two temperatures was measured in bioassays with *Galleria* larvae at 15 °C and was documented as the number of nematodes that infected each larva. Bioassays used single larvae in stoppered plastic vials containing 25 ml of sand and 200-258 infective nematodes. The larvae were replaced in each vial every 4 days until infections ceased. Survival of both nematode species was high in sand at 5 °C. However, the numbers that infected the *Galleria* larvae at 15 °C after storage at 5 °C produced a curve. That is, fewer nematodes infected as the period of storage increased, but after a time, the numbers that invaded the *Galleria* larvae increased and eventually attained the original level. Survival of the Nashes strain was tested at 15 °C and the number of infective juveniles declined until few could be extracted from the sand after 16 weeks storage. The number of nematodes per host showed a similar pattern. It appears that storage at 5 °C induces most of the nematodes into a state in which they lose their ability to parasitise a host. Infectivity is regained after a period of cooling. These effects of storage temperature on infectivity were not reflected in the data for mortality of the insects, which generally remained high in all tests. These studies should be repeated and extended to other species because entomopathogenic nematodes are frequently stored at low temperatures before use in experiments or in biocontrol programmes. This practice may affect virulence of the parasites and the period of storage may be critical.

RÉSUMÉ

Effets du stockage à basse température sur la survie et le pouvoir infestant de deux espèces de Steinernema *(Nematoda : Steinernematidae)*

La survie de deux souches britanniques de Steinernématides — l'une appartenant à *Steinernema feltiae* (Filipjev) [= *S. bibionis* (Bovien)], l'autre à une espèce non décrite désignée comme *Steinernema* sp. (souche Nashes) — est évaluée après stockage des juvéniles infestants dans du sable stérile à 5 °C et 15 °C. Cette survie est mesurée par référence à la mobilité des animaux (possibilité de récupération grâce aux « mini-plateaux » de Whitehead). Le pouvoir infestant est mesuré, après stockage aux deux températures, par bioessai à 15 °C en utilisant des larves de *Galleria* et en se fondant sur le nombre de nématodes infestant chaque larve. Chaque bioessai comprend une seule larve placée dans une fiole plastique bouchée contenant 25 ml de sable et 200 à 258 nématodes de stade infestant. Les larves sont remplacées tous les 4 jours jusqu'à cessation de l'infestation. La survie des deux espèces de nématode s'est révélée élevée dans le sable placé à 5 °C. Cependant, les nombres de nématodes infestant les larves de *Galleria* à 15 °C après stockage à 5 °C se répartissent sur une courbe traduisant le fait que la valeur de l'infestation décroît lorsque la période de stockage s'allonge, mais qu'après un certain temps, cette valeur se relève et peut même atteindre le niveau initial. La survie de la souche Nashes, testée à 15 °C, et le nombre de juvéniles infestants décroissent au point que peu d'entre eux peuvent être récupérés après un stockage de 16 semaines. Le nombre de nématodes par hôte suit un schéma similaire. Il apparaît que le stockage à 5 °C induit chez la plupart des nématodes un état particulier faisant perdre la capacité d'infester l'hôte. Le pouvoir infestant est récupéré après une certaine période de froid. Les conséquences de la température de stockage sur le pouvoir infestant ne sont pas reflétées par les données relatives à la mort des insectes, celle-ci étant généralement élevée dans tous les tests. Ce type d'étude devrait être répété et étendu aux autres espèces car les nématodes entomopathogènes sont fréquemment stockés à basse température avant utilisation pour des expériences ou des programmes de lutte biologique. Une telle pratique peut affecter la virulence des parasites et la durée du stockage devenir critique.

Entomopathogenic steinernematid nematodes are obligate associates of insects and have a mutualistic association with bacteria belonging to the genus *Xenorhabdus*. Infective third stage juveniles termed dauer larvae harbour bacterial cells and release them after they gain access to the haemocoele. The bacteria proliferate, killing the insect, and provide optimal conditions for reproduction of the nematodes. The nematodes go through several generations and usually produce thousands of new infective stages a few weeks after infection. These enter the soil and survive, without feeding, until they either infect another insect or die. Persistence in the soil will be affected by a number of biotic and abiotic factors, with temperature being a major influence. Most studies use mobility of infective juveniles or infectivity (assessed by the percent mortality of a group of test insects) as an indicator of nematode persistence (for example, Saunders & All, 1982; Molyneux, 1985; Ishibashi & Kondo, 1986*b*). For population dynamics studies, it is essential to know the number of nematodes that survive and infect each host rather than the mortality that they cause in the host population (Hominick & Reid, 1990). Therefore, we documented the effects of low temperature on survival and infectivity of two British steinernematids in sterile sand. Survival was assessed by the numbers of nematodes that can be extracted by Whitehead trays and infectivity by the numbers that establish in *Galleria* larvae.

Materials and methods

The nematodes used were isolated from British soil and had been cultured through several generations in *Galleria mellonella* larvae. Following the specific designations suggested by Poinar (1989), one is *Steinernema feltiae* (Filipjev) [= *S. bibionis* (Bovien)], designated Site 42 strain, the other is a sibling of this species, designated *Steinernema* sp. (Nashes strain). Details of these nematodes are in Fan and Hominick (1991). In all experiments, nematodes were cultured in *Galleria* larvae at 20 °C, and infective juveniles were used within one week of emergence.

The method used to infect single *Galleria* larvae was based on that of Molyneux (1985) and has been described (Fan & Hominick, 1991). It involved placing 25 ml of sand (washed, autoclaved and oven-dried), moistened with 1 ml of tap water, into a 30 ml plastic universal vial. The nematodes were added in 1 ml of tap water, introduced into a centrally-made hole which was then obliterated by shaking. A *Galleria* larva was placed on the sand surface, the lid was screwed on, and the vial was inverted.

Survival and infectivity of the Site 42 Strain were documented at 5 °C, with a mean of 215 (range 200-226) infective nematodes per vial. Vials were divided into five groups, each with fifteen replicates. Each group was wrapped with water-saturated tissue and placed in a sealed plastic bag at 5 °C. On Day 0 (control) and Weeks 1, 2, 3 and 4, a group was removed and treated as follows : *1)* A *Galleria* larva was placed in each of five vials, which were incubated at 15 °C. Every four days, a fresh larva was added until no more deaths occurred. The numbers of nematodes in each cadaver were counted, as detailed in Fan and Hominick (1991). *2)* Another five vials were emptied individually into five miniature Whitehead trays (Whitehead & Hemming, 1965). At 24 h intervals at 20 °C, the water was replaced and the number of nematodes that had emerged from the sand was recorded. This was repeated until nematodes ceased to emerge. *3)* The sand in the remaining five tubes was emptied into five beakers and the nematodes were extracted using a sucrose extraction method (Saunders & All, 1982) and counted.

Survival and infectivity of the Nashes Strain was studied at both 5 °C and 15 °C. Three hundred vials containing a mean of 250 (range 239-258) infective nematodes were prepared and divided into fourteen groups of 20 in plastic bags. Seven groups were incubated at 5 °C, seven at 15 °C, and one served as the Day 0 Controls. On Day 1 and Week 2, 4, 6, 8, 12 and 16, one group was removed from each temperature. Then, ten replicates of the two groups were exposed to *Galleria* larvae individually at 15 °C. Fresh larvae were added every four days until none were killed. Five replicates were extracted by the miniature Whitehead trays at 20 °C and the remaining five were subjected to sucrose extraction. Results were documented as in the first experiment.

Results

Survival of *S. feltiae* (Site 42) in sand at 5 °C is documented in Figure 1. Immediately after inoculation

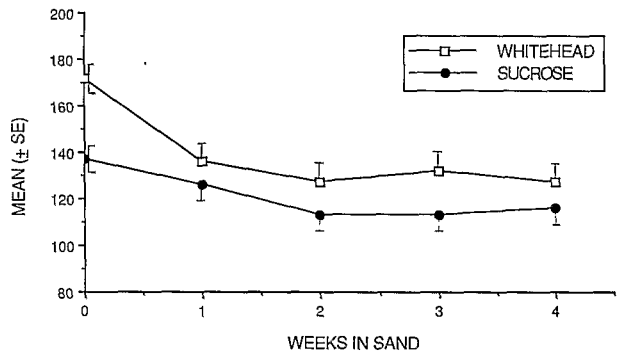

Fig. 1. Mean and standard error for the number of infective juveniles of the Site 42 strain of *Steinernema feltiae* (= *S. bibionis)* recovered after storage in moist sand at 5 °C for up to four weeks. Recovery by Whitehead trays at 20 °C or sucrose extraction, 200-226 nematodes per 25 ml sand, 5 replicates per treatment.

(Day 0), approximately 80 % of the nematodes were recovered by the miniature Whitehead trays, which indicates the efficiency of the technique. A significant reduction in the numbers of nematodes recovered occurred on Week 1 ($t = 4.207$; $p < 0.005$). Thereafter, there was no significant difference between the mean number recovered on Week 1 and the mean numbers on Weeks 2, 3, and 4 (ANOVA; $F = 0.37$; $p > 0.05$). A similar pattern of recovery was obtained by the sucrose method, but the numbers were fewer than with the Whitehead tray method.

Infectivity of the nematodes, as measured by mean number per *Galleria*, showed a different trend compared to survival (Fig. 2). A remarkable decrease in numbers per host occurred after the nematodes had been stored in sand at 5 °C for one week. Then, a significant and surprising rise in the number per host was recorded after the infective juveniles had been stored in sand for four weeks ($t = 3.986$; $p = 0.016$). Thus, on Week 4, the mean number (± se) of nematodes per host (53 ± 10) was not significantly different from the mean number recorded on Day 0 ($t = 0.069$; $p = 0.947$). This change in infectivity was not reflected in data for mortality of the insects. All the insects were parasitised in every assessment, so mortality remained at 100 % throughout the experiment. Figure 2 also shows the accumulated mean number of nematodes per host after 6-11 consecutive 4-day exposures. The results were more variable, but the same trend occurred. There was no significant difference between the means in the single and consecutive exposures on Day 0 ($t = 0.76$; $p = 0.472$) of Week 4 ($t = 0.888$; $p = 0.404$). Thus, most of the nematodes that could infect did so on the day of inoculation or after four weeks' storage.

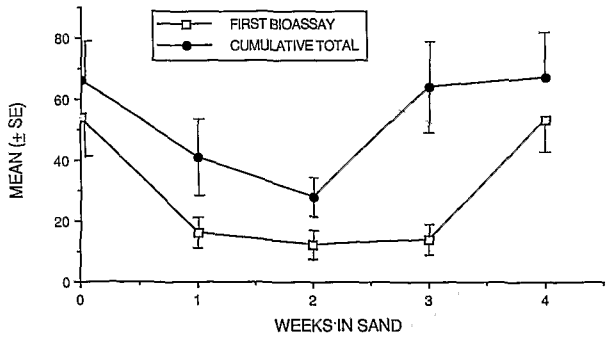

Fig. 2. Mean and standard error for the number of nematodes of the Site 42 strain of *Steinernema feltiae (= S. bibionis)* recovered from each *Galleria* larva in the bioassay at 15 °C, after the infective nematodes had been stored in moist sand at 5 °C for up to four weeks. Five replicates per treatment, 200-226 nematodes per 25 ml sand, one *Galleria* larva per vial. Lower line shows data for first bioassay, upper line shows cumulative totals resulting from 6-11 successive 4 day exposures to *Galleria* larvae until infections ceased.

Survival of *Steinernema* sp. (Nashes strain) in sand, as assessed by the Whitehead tray method, was much better at 5 °C compared to 15 °C (Fig. 3). On Day 0, a mean of 211 ± 11 (se) nematodes was recovered, giving an efficiency of 84 % for the method. A mean of 162 ± 11 nematodes was still extracted from the sand after storage at 5 °C for 16 weeks, compared to only 6 ± 2 nematodes from the sand stored at 15 °C for the same length of time. Sucrose extraction produced results with similar trends, though slightly lower numbers were recovered compared to the Whitehead tray method when the nematodes had been stored at 5 °C, while higher numbers were obtained after storage at 15 °C. Significant differences were found in the numbers obtained by the two extraction techniques on Week 16 after storage at 5 °C ($t = 4.556$; $p = 0.003$) but the difference after storage at 15 °C was not significant ($t = 1.358$; $p = 0.223$).

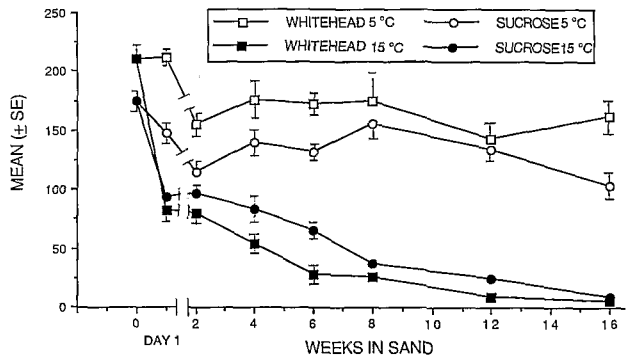

Fig. 3. Mean and standard error for the number of infective *Steinernema* sp. (Nashes Strain) juveniles recovered after storage in moist sand at 5 °C or 15 °C for up to 16 weeks. Recovery by Whitehead trays at 20 °C or sucrose extraction, 239-258 nematodes per 25 ml sand, 5 replicates per treatment.

Infectivity assessed by mortality of the hosts showed a slight trend to decrease as storage time at 5 °C increased and a more definite trend for storage at 15 °C (Fig. 4). However, the dramatic decrease in the number of surviving nematodes after storage at 15 °C was not reflected by the mortality that they caused for the insects. Thus, although a mean of only six infective juveniles was recovered from the sand after storage for sixteen weeks at 15 °C, 40 % of the *Galleria* larvae were still killed.

Infectivity assessed by the mean number of parasites per host showed very different patterns compared to assessment by mortality of the insects (Fig. 5). There were significant differences in both mean numbers of nematodes that infected the larvae and in the pattern of recovery for the nematodes stored at the two temperatures. Infectivity after storage at 15 °C decreased rapidly so that by Week 16, a mean of only two nematodes was

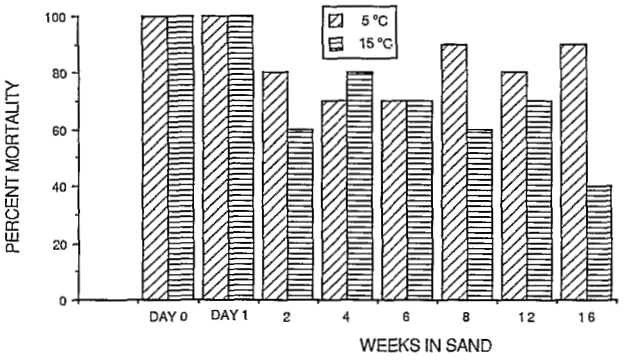

Fig. 4. Percent of *Galleria* larvae killed after 96 hr by infective *Steinernema* sp. (Nashes Strain) juveniles which had been stored in moist sand at 5 °C or 15 °C for up to 16 weeks. Bioassays at 15 °C, n = 10, 239-258 nematodes per 25 ml sand, one *Galleria* larva per vial.

recovered from each *Galleria* larva. This is less than 3 % of the number that established on Day 0 (Fig. 5 A). In contrast, infectivity at 15 °C after storage at 5 °C produced a curve showing a rapid decline followed by a surprising increase, as it did in the previous experiment using *S. feltiae* (Site 42). Thus, infectivity decreased rapidly to a mean of 10 nematodes/larva after four week's storage and then gradually increased to a mean of 70 nematodes/larva after storage for 16 weeks (Fig. 5 B). Consequently, infectivity of the nematodes after 16 week's storage at 5 °C returned to the same level as on Day 0 (80 nematodes/larva).

Most of the nematodes stored at 15 °C for various lengths of time penetrated during the first exposure to *Galleria* and additional exposures to fresh larvae did not result in significantly higher infections (Fig. 5 A). However, for the nematodes which were stored at 5 °C, only Day 0 and Week 16 bioassays showed no significant differences between the mean numbers which established after one exposure and the accumulated totals after up to eleven consecutive exposures (Fig. 5 B). Thus, when Nashes nematodes were stored at 5 °C, their infectivity decreased rapidly but returned to the level observed for " fresh " nematodes after sixteen week's storage. Furthermore, after Day 1 and before Week 16, nematodes gradually recovered infectivity if allowed extended exposure times to *Galleria* larvae. However, over Weeks 2-12, the nematodes never attained the infection levels of those on Day 0 and 1 or Week 16.

Results documented elsewhere (Fan & Hominick, 1991) show that the percent of a dose of nematodes that establish in *Galleria* in our bioassays under conditions ideal for infection is within the range 30-40 %, and is independent of the dose applied. The experiments documented here go farther in that the number of surviving nematodes in sand has been assessed, so that

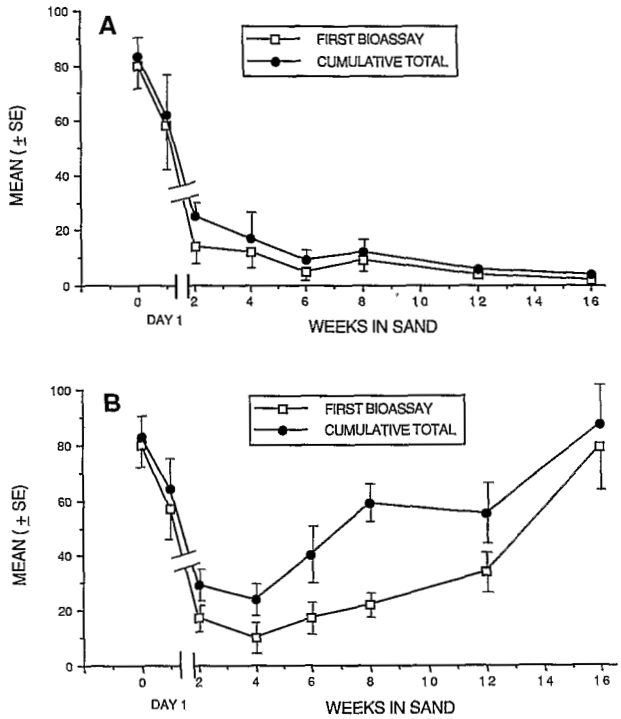

Fig. 5 A, B. Mean and standard error for the number of *Steinernema* sp. (Nashes Strain) nematodes recovered from each *Galleria* larva in the bioassay at 15 °C, after the infective nematodes had been stored in moist sand at 15 °C (A) or 5 °C (B) for up to 16 weeks. Ten replicates per treatment, 239-258 nematodes per 25 ml sand, one *Galleria* larva per vial. Lower line shows data for first bioassay, upper line shows cumulative totals resulting from 4-11 successive 4 day exposures to *Galleria* larvae until infections ceased.

the total number of nematodes which infected successive *Galleria* larvae can be expressed as a percentage of the surviving mobile nematodes (those extracted with the Whitehead trays). Figure 6 presents the data and shows that there was little difference between the percent of Site 42 nematodes that infected on Day 0 (Fig. 6 A) and the percent of Nashes that infected on that day (Fig. 6 B). Then, when the nematodes were stored at 5 °C, there was a drop in infectivity after several week's storage, followed by a steady increase. Thus, by Week 4, 52.5 % of the surviving Site 42 nematodes were infective (Fig. 6 A). The rate of increase for Nashes nematode was slower, but by Week 16, 54 % of the survivors were infective (Fig. 6 B). Therefore, it appears that storage of the nematodes at 5 °C does not dramatically affect survival (Figs. 1, 3) but does significantly affect the infectivity of the survivors (Fig. 6). Infectivity of the surviving Nashes nematodes after storage at 15 °C had a similar pattern compared to the one obtained at 5 °C, except for the unexplained abnormality on Day 1

(Fig. 6 B). However, while survival at 15 °C was much poorer compared to 5 °C (Fig. 3), those nematodes that did survive were highly infective. Thus, after six weeks' storage at 15 °C, infectivity of the survivors began to increase and eventually reached 74 % on Week 16 (Fig. 6 B).

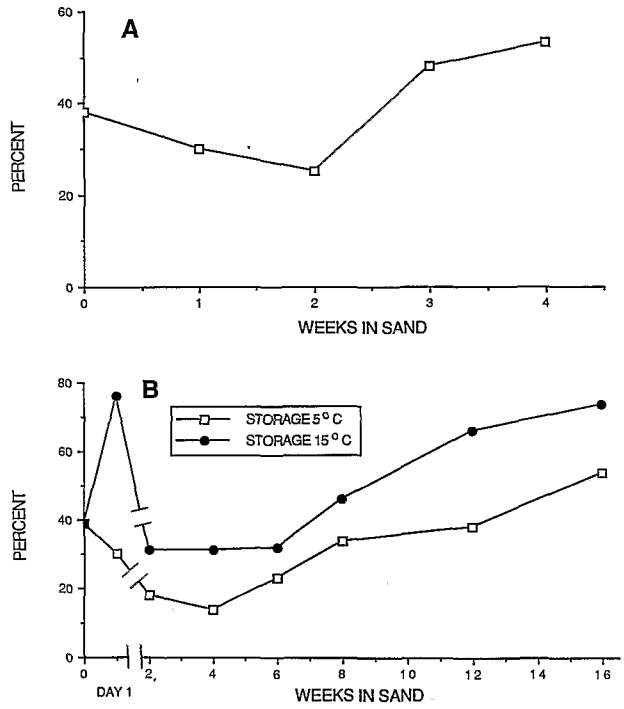

Fig. 6 A, B. Infectivity of the surviving nematodes. The cumulative mean numbers that established in *Galleria* larvae (upper curves in Figs 2 & 5) are expressed as a percentage of the mean numbers extracted by the Whitehead trays. A : Site 42 strain of *Steinernema feltiae (= S. bibionis)* after storage at 5 °C, data from Figures 1 & 2; B : *Steinernema* sp. (Nashes Strain) after storage at 5 °C or 15 °C, data from Figs 3 and 5.

Discussion

The most important discovery in this study is that the infectivity of juveniles after being stored in sand at 5 °C, when measured by the numbers which could infect a highly susceptible host at optimal temperatures, showed a trend to decrease and then increase with passing time instead of an expected continuously decreasing trend. The same pattern was found for both *Steinernema* species, the only difference being the time lapse between the moment when the nematodes showed reduced infectivity and the moment when they regained it. This possibly represents a biological difference between the two species. Because this curve was not observed for nematodes stored in sand at 15 °C (a temperature approximating mean summer soil temperatures 5 cm deep in Britain; Hominick & Briscoe, 1990), the cold temperature appears to induce most of the nematodes into a state in which they lose their ability to parasitise a host. However, their movement appears unaffected because the extraction results from the sand showed continued high rates of recovery. Even if the nematodes were allowed access to successive *Galleria* larvae for up to 44 days, they never attained the infection levels recorded at the start and end of the experiment. It therefore appears that an obligatory period of cooling is required before all the nematodes regain their infectivity. Such a response to low temperature must have survival benefits for the population. Perhaps it is advantageous for most infective stages to persist in the environment at low temperatures rather than to infect a host. At least some individuals of the Nashes strain are physiologically capable of infecting *Galleria* larvae at 5 °C (Fan & Hominick, 1990). In any case, such studies should be repeated and extended to other species because entomopathogenic nematodes are frequently stored at low temperatures before use in experiments or in biocontrol programmes. This may affect virulence of the parasites and the period of storage may be critical.

Survival of infective juveniles has been assessed by Whitehead sieves (Molyneux, 1985; Ishibashi & Kondo, 1986*b*, 1987), sucrose extraction-centrifugal flotation (Saunders & All, 1982; Ishibashi & Kondo, 1986*b*, 1987) and parasitisation of insects by nematodes (Molyneux, 1985). Saunders and All (1982) showed that Baermann funnel extraction (which works on the same principle as the Whitehead tray) was superior to centrifugal flotation for recovering *N. carpocapsae* from sand. Ishibashi and Kondo (1986*a*) obtained opposite results with the same species and felt that the nematodes were probably in a quiescent state during their experiments. Their reasoning was that Whitehead trays require the nematodes to be active while centrifugation does not. In our hands, the sucrose extraction method generally recovered fewer nematodes, which supports the results of Saunders and All (1982). However, we also found that more nematodes were extracted by the sucrose method than by the Whitehead trays after the nematodes had been stored at 15 °C. At this temperature, the *Steinernema* sp. (Nashes) nematodes recovered from the sand appeared transparent and the degree of transparency appeared to increase with storage time. Also, more transparent nematodes were recovered when the sucrose extraction method was used. Therefore, at 15 °C, the lower number of nematodes obtained by the Whitehead trays compared to sucrose extraction was probably related to their decreased motility or inability to migrate out of the sand because of depleted energy stores. However, the numbers were only marginally smaller than those recovered by the sucrose method and the bioassays showed that

few were able to infect *Galleria* larvae. Thus, Ishibashi and Kondo's (1986*a*) claim that *S. feltiae* (= *S. carpocapsae*), which had been stored at 25 °C, were in a state of quiescence is questionable. It is more likely that the nematodes had used up much of their nutrient reserves at the high temperature. Indeed, Molyneux (1985) felt that *S. feltiae* (= *S. carpocapsae* Agriotos) infective juveniles showed poor survival at higher temperatures because of rapid utilisation of limited energy stores. By contrast, *S. glaseri* survived extremely well at elevated temperatures, so that after 32 weeks in sand at 28 °C, half of the nematodes applied could still be recovered. This was because the *S. glaseri* KG strain became quiescent in sand, characterised by a coiled posture even at 23 °C, thus conserving energy (Molyneux, 1985).

Temperature is an important factor in the life of entomopathogenic nematodes, affecting mobility, survival, infectivity, development and reproduction. The present study with Whitehead trays provides results similar to those of Molyneux (1985) for *S. feltiae* (= *S. carpocapsae*) and *S. glaseri*, whereby colder temperatures result in longer survival. Infectivity measured by percent mortality of insects in a bioassay is a more reliable indicator than mobility for measuring nematode virulence (Molyneux, 1985; Ishibashi & Kondo, 1986*b*). However, if the population dynamics of these nematodes are to be understood, it is essential to document numbers of nematodes rather than percent mortality that they cause in the host population (Hominick & Reid, 1990). This is well illustrated by the present experiments where mortality of the *Galleria* larvae was not indicative of the survival of the nematodes as assessed by movement through a Whitehead sieve. Molyneux (1985) showed that parasitisation of *Lucilia cuprina* declined with time in a similar trend to their survival as assessed by mobility. This undoubtedly reflects the different insects used in the assays, as *Galleria* larvae are highly susceptible to entomopathogenic rhabditids. Nevertheless, bioassays utilising *Galleria* larvae and counting the numbers of nematodes that establish in each appear to offer great promise for elucidating the population dynamics of these nematodes (Fan & Hominick, 1991).

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