ADAPTATION TO TEMPERATURE IN ENTOMOPATHOGENIC NEMATODES

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ADAPTATION TO TEMPERATURE IN ENTOMOPATHOGENIC NEMATODES

By

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ABSTRACT

The effects of recycling over a two-year period at temperatures from 10- 25 °C were studied in four strains of entomopathogenic nematodes: *Steinernema carpocapsae* All strain, *Steinernema feltiae* NF strain, *Steinernema feltiae* Umeå strain and *Steinernema riobravis* TX strain. The main objectives of this investigation were to study the capacity for, and mechanisms involved in, thermal adaptation of a native (NF strain) boreal steinernematid nematode and to compare it with strains of entomopathogenic nematodes originating from another boreal region (Sweden; Umeå strain), temperate zone (All strain) and subtropical zone (TX strain).

On the basis of morphometry and restriction fragment length polymorphism in ribosomal DNA spacer, a nematode that was isolated from soil near St. John's, Newfoundland, Canada, was designated as a new strain, NF, of *S. feltiae*. To determine whether taxonomic separations of entomopathogenic nematodes could be accomplished on the basis of physiological properties other than DNA profiles, cellulose acetate electrophoresis was used to separate isozymes of eight enzymes in infective juveniles of five strains of *Steinernema*. These strains comprised the four listed above, together with *S. feltiae* L1C strain, isolated 14 years previously from Newfoundland. Based on comparisons of the relative electrophoretic mobilities (μ) of the isozymes, it was concluded that the five isolates could be distinguished. This finding suggests that cellulose acetate electrophoresis could be an important taxonomic tool for the identification of isolates of steinernematids, provided that a combination of enzymes is used.

The capacities for high temperatures and freezing tolerance were determined and found to be affected by the temperatures at which recycling was carried out. In all the isolates, the UT_{50}, the temperature at which 50% of the infective juveniles died, increased with increase in

recycling temperature. Freezing tolerance, as measured by times at which 50% of the infective juveniles were killed (Lt_{50}) by a -5 °C regime, was reduced at higher recycling temperatures. Boreally adapted *S. feltiae* was more cold tolerant than *S. carpocapsae* and *S. riobravis*, but its capacity to withstand freezing was reduced by recycling at warmer temperatures. The greatest capacity for heat tolerance was observed in *S. riobravis*, while *S. carpocapsae* displayed an intermediate degree of tolerance to high temperatures.

Infectivity was found to be modified by the recycling temperatures. This was examined by measuring LD_{50} values in *G. mellonella* larvae at bioassay temperatures from 5 to 25 °C. Only the infective juveniles of *S. feltiae* that had been recycled at 10 °C infected and killed the insects at a 5 °C bioassay temperature, whereas *S. carpocapsae* and *S. riobravis* were infective at 10 °C only when the recycling temperatures were ≤ 20 °C. The infectivity of the two strains of *S. feltiae* at 10 or 15 °C was compromised by propagating them at warmer temperatures (20-25 °C). The *S. feltiae* Umeå strain displayed an impaired capacity to infect hosts at warm temperatures (20- 25 °C) when recycled at colder (≤15 °C) temperatures.

The pattern of isozymes was modified according to the recycling temperature regime. In all four isolates, isozyme banding patterns of malate dehydrogenase (MDH), mannose-6-phosphate isomerase (MPI) and phosphoglucomutase (PGM) were affected by recycling or storage temperature. The two strains of *S. feltiae* synthesized additional isozymes of MPI and MDH or PGM in response to cold temperatures, while *S. carpocapsae* All strain synthesized three isozymes of MDH in response to warm temperatures. Modifications of isozyme synthesis may constitute a temperature adaptation mechanism for these nematodes.

In all four isolates, the kinetic properties of glucose-6-phosphate dehydrogenase and

hexokinase were affected by the recycling temperature. At each assay temperature (5- 35 °C), the maximum specific activity of both the enzymes was greater in the nematodes that had been recycled at lower temperatures than in those reared at higher temperatures. In three enzyme - nematode isolate combinations, the lowest K_m values occurred in nematodes that had been recycled at the lower temperatures.

Using gas liquid chromatography, it was determined that the composition of fatty acids in total lipids and phospholipids changed adaptively with recycling temperatures. In all four isolates, the unsaturation indices of total lipids and phospholipids were increased as temperature decreased. This was due to an increase in polyunsaturated fatty acids a with concomitant decline in the proportion of saturated fatty acids, especially palmitic (16:0) and/or stearic (18:0) acids. The increase in polyunsaturated fatty acids at reduced temperatures was attributed to significantly greater percentages of linoleic acid (18:2) in total lipids and phospholipids. In all except *S. riobravis*, this was augmented by increased proportions of eicosapenic acid (20:5*w*3) at 5 °C.

Together, these results suggest that the various recycling temperatures influenced the capacities of the entomopathogenic nematodes for temperature tolerance and infectivity at an organismal level. At the physiological level, the synthesis of isozymes, enzyme kinetics, and composition of fatty acids in lipids were modified by the recycling temperatures in an apparently adaptive fashion. The degree to which the various physiological changes provide an underlying basis for those at the organismal level is discussed.

This thesis is dedicated to the memory of

my father, aunt and uncle who were

the continuous source of

inspiration for me

in every walk

of life.

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TABLE OF CONTENTS

page

LIST OF TABLES

CHAPTER 2.

CHAPTER 3.

CHAPTER 4.

CHAPTER 5.

CHAPTER 6.

CHAPTER 7.

LIST OF FIGURES

CHAPTER 2.

CHAPTER 3.

CHAPTER 6.

CHAPTER 1

GENERAL INTRODUCTION

1.1. The need and potential for biological control

With the development of synthetic organic pesticides immediately after World War II, the use of modern insecticides to control insect pests has been a necessary part of crop production and prevention of insect-related health problems. However, extensive use of pesticides has posed several problems, such as persistence of pesticidal residues in the food-chain, insect resistance to chemicals, adverse effects on beneficial organisms, pest resurgence, health and environmental risks (Ehlers, 1996; Metcalf and Luckmann, 1994). In order to protect the environment and improve the productive capacity of agriculture, the use of pesticides should be reduced/eliminated and alternative methods for control of insect pests developed.

Biological control is a good alternative method for pest management and has been considered for use since the last century (Metcalf and Luckmann, 1994). The principal advantages of this method are: suppression of the insect pest species below the economic injury level, self- perpetuating characteristics and reduced environmental and health risks (Ehlers, 1996; Ehlers and Hokkanen, 1996; Metcalf and Luckmann, 1994).

1.2. Nematodes as potential biocontrol agents

Insect control by using their natural nematode parasites occupies a prominent position among the biological control agents against insects proposed to date. According to the survey conducted by Lisansky and Coombs (1994), nematodes share 13.3% of the current $75m biopesticide market and there is a growing interest among farmers for the

use of nematodes as biopesticides against insect pests infesting citrus, turf grass and mushrooms in the U.S.A. and Japan as well as against green house pests in Europe (P. Grewal, personal communication; Ehlers, 1996; Gaugler and Hashmi, 1996; Grewal and Smith, 1995; Klein, 1993). Although nematodes belonging to 19 families are recognised as facultative or obligate parasites of a wide range of insect orders (Poinar, 1979), only the members of three families, Mermithidae, Heterorhabditidae and Steinernematidae, have been considered to be potentially effective biocontrol agents. (Bedding *et al.*, 1993; Gaugler and Kaya, 1990; Petersen, 1985).

1.2.1. Mermithidae

The mermithids are a family of nematodes that parasitize arthropods. Over 200 species of mermithids from 32 genera have been reported as parasites of insect species (Nickle, 1981; Petersen, 1984; Poinar, 1979). The newly-hatched infective juveniles (syn. pre-parasites) of these nematodes enter the hemocoel of insect hosts by penetration of the host's cuticle (Shamseldean and Platzer, 1989). Within the hemocoel, nutrients are directly absorbed from the hemolymph by transcuticular uptake and stored in the nematode's trophosome (Gordon and Burford, 1984) for subsequent utilization by the post-parasitic free-living stage. Upon completion of parasitic development, the nematodes emerge as free-living stages (postparasites) from the host by rupturing the host's exoskeleton. This rupturing of the cuticle is lethal to the host. The post-parasitic stage enters the soil/sediment, matures, mates and lays eggs to complete the life cycle (Petersen, 1985).

During the 1970s, most of the fundamental and applied research on insect parasitic nematodes was devoted to this family, especially to the species that are parasitic to mosquitoes (Petersen, 1985). *Romanomermis culicivorax* Ross and Smith, 1976, was widely regarded as a potential biocontrol agent for mosquitoes (Petersen, 1984). Despite its biocontrol potential, research on *R. culicivorax* has declined during the last decade, because of the inability to culture mermithids in vitro and consequent difficulty in obtaining sufficient numbers of infective juveniles of the nematode at a commercially-feasible cost for field applications. Poor storage and transport capabilities of these nematodes were additional drawbacks to their commercialization (Kaya *et al.*, 1993).

1.2.2. Heterorhabditidae and Steinernematidae

Because of the failure of attempts to develop mermithid nematodes as insect biocontrol agents, most of the subsequent research during the 1980s and 1990s has been focused on nematode species from two families: the Steinernematidae and Heterorhabditidae. This is because these nematodes are easily mass produced, widely distributed in nature, possess broad host ranges and kill the host within 48 hours. They are readily mass produced, harmless to economically important insects, vertebrate animals, and plants, and compatible with many pesticides (Bathon, 1996; Bedding *et al.*, 1993; Gaugler and Kaya, 1990). The steinernematid and heterorhabditid nematodes are called entomopathogenic nematodes because they are symbiotically associated with bacteria which play an important role in pathogenesis, nematode nutrition and development

(Boemare *et al.*, 1993; Popiel and Hominick, 1992).

1.2.2.1. Biology of heterorhabditid and steinernematid nematodes

The third stage (dauer) infective juveniles are thought to be non-feeding stages, generally found in the soil and carrying species- specific bacteria of the genera *Xenorhabdus* (in f. Steinernematidae) and *Photorhabdus* (in f. Heterorhabditidae) in their intestines. Infective juveniles of steinernematids enter their host's hemocoel through natural openings such as the mouth, anus and spiracles (Poinar, 1990), whereas those of heterorhabditids also enter their host's hemocoel through natural openings or by penetrating directly through the exoskeleton (Bedding and Molyneux, 1982). Once in the hemocoel, each infective juvenile releases the mutualistic bacteria from its anus. The bacteria release toxins to kill the insect host, usually within 48 hours at 20- 25 °C. Bacteria also produce antibiotics to preserve the insect cadaver against putrification and reproduce within the cadaver, providing a nutritional milieu for nematode development and reproduction (Poinar, 1990). Depending upon the availability of food, nematodes produce 2-3 generations within the insect cadaver and emerge as infective juveniles to search for new hosts. The life cycle of steinernematid nematodes is generally completed within 7-10 days at room temperature (ca. 25 °C), whereas for heterorhabditid nematodes 12-15 days are required to complete their life cycle (Kaya *et al.*, 1993). The basic difference between the reproduction of steinernematid and heterorhabditid nematodes is that the infective juveniles of *Steinernema* develop into males and females which mate and

reproduce. while those of *Heterorhabditis* develop into hermaphroditic adults. The subsequent generations of this nematode develop into unisexual males and females to effect reproduction (Poinar. 1990).

1.2.2.2. Taxonomy of Heterorhabditidae and Steinernematidae

Based on classical morphological and biochemical methods, there are currently 18 species of *Steinernema* and three species of *Heterorhabiditis* which have been reported to infect insects (Akhurst, 1995: Popiel and Hominick, 1992), several existing as geographically distinct strains (*Steinernema* = 63 strains and *Heterorhabiditis* = 38 strains) (Poinar. 1990). Although classical morphological methods have been successfully used for the identification of entomopathogenic nematodes at the level of genus and species (Poinar. 1990), the ability to be able to identify strains is an important aspect of the overall research and development involving this family of nematodes. Curran (1990) concluded that DNA sequence analysis constitutes the preferred molecular method for discriminating between categories of entomopathogenic nematodes below the species level. To facilitate this, DNA amplification techniques by polymerase chain reaction (PCR) have been developed for entomopathogenic nematodes (Liu and Berry, 1995).

Protein electrophoresis could be a useful taxonomic tool, enabling nematologists to more rapidly and cost effectively discriminate between species. Starch gel electrophoresis revealed an array of esterase and alkaline phosphatase isozymes in *Steinernema glaseri* that were qualitatively and quantitatively different from that in

Steinernema carpocapsae (Sherman and Jackson. 1963). This same procedure, adapted to separate isozymes of several enzymes, was used by Akhurst (1987) to distinguish between 22 strains representing 3-8 species of *Heterorhabditis*. Polyacrylamide gel electrophoresis was used to discriminate between five isolates belonging to four species of *Steinernema* on the basis of their esterase isozyme patterns (Sha, 1985) and ten isolates belonging to four species of this genus according to their total protein, nonspecific esterase and alkaline phosphatase patterns (Kozodoi *et al.*, 1986). Poinar and Kozodoi (1988) showed that two morphologically similar steinernematids, *S. glaseri* and *Steinernema anomali*, possessed dissimilar and distinctive enzyme patterns, as revealed by polyacrylamide gel electrophoresis.

In 1983, a steinernematid nematode (*Steinernema feltiae* L1C strain) was isolated from a site close to St. John's, Newfoundland, Canada (Finney-Crawley, 1985). However, the original collection site has been paved over. Therefore, in an attempt to find a cold-adapted species of an entomopathogenic nematode, a survey of potential field sites was carried out during the Summer 1993. As a part of this survey, soil samples were collected and a steinernematid nematode was isolated from soil in an organic garden close to St. John's, Newfoundland, Canada, using *Galleria* bait traps (Woodring and Kaya, 1988). Based on classical morphological methods, restriction fragment length polymorphisms in ribosomal DNA and protein electrophoresis (cellulose acetate electrophoresis), this newly isolated nematode was subsequently identified as a new strain, NF, of *S. feltiae* (Jagdale *et al.*, 1996). In addition to the NF and L1C strains of *S. feltiae*, the present study

embodies three additional isolates of *Steinernema*: *S. feltiae* Umeå strain, *S. carpocapsae* All strain and *S. riobravis* TX strain.

1.2.2.3. Factors affecting the success of entomopathogenic nematodes

Generally, entomopathogenic nematodes proved to be very effective in infecting and killing a variety of insects under optimal laboratory conditions (petri dish bioassays) where host contact is predictable (Caroli *et al.*, 1996). However, the field evaluation of these nematodes against several soil-dwelling and foliar-feeding insects produced inconsistent results (Begley, 1990; Gaugler, 1988; Klein, 1990). Survival and field efficacy of these nematodes may be affected by several biotic and abiotic factors (Kaya, 1990; Kaya and Koppenhöfer, 1996; Kaya and Thurston, 1993; Popiel and Hominick, 1992; Richardson and Grewal, 1994). Therefore, knowledge of these factors is important in the successful use of entomopathogenic nematodes in pest management programmes.

1.2.2.3.1. Biotic factors

Natural enemies of entomopathogenic nematodes may affect their survival and infectivity in the field. Such natural enemies include nematophagous fungi (Poinar and Jansson, 1986; Timper and Kaya, 1989), nematode trapping fungi (Poinar and Jansson, 1986), microsporidian parasites (Poinar, 1988), predatory nematodes and arthropods (Epsky, *et al.*, 1988; Kaya and Koppenhöfer, 1996). Biotic factors include competition between two species of entomopathogenic nematodes and their interaction with other

microbial control agents (Kaya, 1990; Kaya and Koppenhöfer, 1996; Kaya and Thurston, 1993; Popiel and Hominick, 1992). Most of the published information on biotic factors is based on laboratory experiments and little is known about their effect on entomopathogenic nematodes under field conditions.

1.2.2.3.2. Abiotic factors

The field efficacy and ability of entomopathogenic nematodes to persist in the soil are affected by several abiotic factors, such as soil pH, soil type, soil moisture, UV light and temperature.

Survival and persistence of natural and applied populations of entomopathogenic nematodes are affected by pH, oxygen levels and porosity of the soil. Kung *et al.* (1990a) observed that the survival and pathogenicity of infective juveniles of *S. carpocapsae* and *S. glaseri* declined slightly as the soil pH decreased from pH 8 to pH 4, but these species completely lost their ability to survive and infect *Galleria mellonella* larvae under alkaline conditions (pH 10). Oxygen appears to be essential for nematode survival, e.g., the survival and infectivity of *S. carpocapsae* and *S. glaseri* decreased as oxygen levels dropped form 20 to 1% (Kung *et al.*, 1990a). The survival and infectivity of entomopathogenic nematodes is affected by soil type and its porosity. Sandy and sandy-loam soils have larger pores (more aeration, i. e. more oxygen) than clay soils. Lower survival was noticed in the infective juveniles of *S. carpocapsae* and *S. glaseri* placed in clay soils (poor aeration) compared to those placed in the sandy or sandy- loam soils

(Kung *et al.*, 1990b). Molyneux and Bedding (1984) reported that the infective juveniles of *S. glaseri* were less infective to the larvae of sheep blowfly, *Lucilia cuprina*, in soils with small pore size. Similarly, infectivity of steinernematid and heterorhabditid nematodes to their hosts was impaired in clay soils (Geden *et al.*, 1985; Molyneux and Bedding, 1984).

Soil texture interacts with soil water potential to influence infectivity under natural conditions (Kaya, 1990; Molyneux and Bedding (1984). In sandy soils, infections of *Heterorhaditis* sp. and *S. glaseri* occurred at water potentials between -0.003 to -0.4 bars, whereas in sandy-loam soil, infections of these nematodes occurred at water potentials between -0.01 to -100 bars (Molyneux and Bedding (1984). The infectivity of *Steinernema kushidai* against larvae of scarabaeid beetles, *Anomala cuprea* (Cuprea chafer beetle), was greater at 30 and 40% than at 20% moisture in soil of an unspecified type (Fujiie *et al.*, 1996). Conversely, infective juveniles of *S. carpocapsae* and *S. glaseri* were more infective to wax moth larvae at ≤ 4% than at ≥ 8 % moisture in sandy loam- soil (Kung *et al.*, 1991).

Although entomopathogenic nematodes have been used to control some foliar feeding insect pests, desiccation has been a critical limiting factor (Begley, 1990; Glazer and Navon, 1990). In order to achieve successful control of defoliating insects, there is a need to maintain appropriate humidity after application or provide adequate moisture (free water) on the leaves for movement of the infective juveniles and infection of the host. For example, the efficacy of *S. feltiae* against leafminers (*Liriomyza huidobrensis*,

Liriomyza bryoniae and *Chromatomyia syngenesiae*) was significantly enhanced at > 90% humidity (Williams and Macdonald, 1995). Desiccation tolerance in soil has also been studied in some entomopathogenic nematodes (Glazer, 1996; Womersley, 1990). It has been demonstrated that *S. carpocapsae* could survive at a wide range of relative humidities provided they were gradually desiccated (Simons and Poinar, 1973; Womersley, 1990). Simons and Poinar (1973) found that more than 90% of *S. carpocapsae* infective juveniles survived after 12 days at 79.5% relative humidity by pre-exposing them to 96% relative humidity for 12 hours and then at 93% relative humidity for another 12 hours. However, Glazer *et al.* (1991) did not find any effect of gradual desiccation on the survival of *S. carpocapsae* All strain and heterorhabditid nematodes. Only 30% of *S. carpocapsae* All strain and none of the heterorhabditids survived after 72 hours exposure to 75% relative humidity. Furthermore, the survival of *S. carpocapsae* and *S. glaseri* decreased as relative humidity decreased from 100 to 25% over 32 days period. Both nematodes survived for 32 days at 100% humidity, but at 25% humidity, *S. carpocapsae* survived for 2 days and *S. glaseri* survived only for 4 hours (Kung *et al.*, 1991).

Sunlight and UV radiation also limit the use of entomopathogenic nematodes as potential biocontrol agents for controlling foliar feeding insects. Gaugler and Boush (1978) reported that the infective juveniles of *S. carpocapsae* incurred a reduction in their infectivity to wax moth larvae and failed to reproduce when exposed to UV radiation and sunlight. Due to the compounding effects of UV light and desiccation, use of

entomopathogenic nematodes in insect pest management has the greatest potential for success in the soil or otherwise cryptic habitats.

Temperature affects the activity, survival, infectivity, reproduction and development of entomopathogenic nematodes in the laboratory as well as in the field (Grewal *et al.*, 1994; Griffin, 1993; Kaya, 1990; Mason and Hominick, 1995; Molyneux, 1985; Steiner, 1996). Although entomopathogenic nematodes are buffered by the soil from environmental extremes (Kaya and Gaugler, 1993), their biological activities may be affected by seasonal and daily temperature fluctuations. Nematodes may move deep into the soil to avoid lethal temperatures (Kaya, 1990) or may move towards the preferred temperature for their normal functioning. The infective juveniles of *S. carpocapsae* have been observed to migrate in response to thermal gradients (Burman and Pye, 1980).

The activity, and thus infectivity, of entomopathogenic nematodes is temperature dependent. For example, steinernematid nematodes were found to be more active than heterorhabditid nematodes at lower temperatures. Two strains of *S. feltiae* were active at temperatures < 7 °C, whereas heterorhabditids were mostly active at temperatures > 10 °C (Molyneux, 1985; Molyneux, 1986). The reduced activity of *Heterorhabditis heliothidis* at 10 °C was responsible for its decreased infectivity against birch casebearer (*Coleoptera serratella*) and honeysuckle leaf roller (*Harpipteryx xylostella*) at the same temperature. However, the infectivities of these nematodes were increased when their activities increased at 24 °C (Finney and Bennett, 1984). Similarly, Blackshaw and Newell (1987) suggested that the reduced infectivity of *H. heliothidis* against *Galleria mellonella* larvae

at 32.3 °C was due to decreased nematode activity.

Survival of entomopathogenic nematodes is affected by storage temperature (Griffin, 1993; Kaya, 1990; Popiel and Hominick, 1992). Entomopathogenic nematodes are stored at low temperatures before being used in biological control programs. Optimum storage temperatures are believed to be species- specific and may be correlated with the temperature profiles characteristic of their original habitats (Georgis, 1990; Molyneux, 1986). Optimum storage temperatures range between 5 and 10 °C for steinernematids and 9 and 15 °C for heterorhabditids (Fan and Hominick, 1991; Georgis, 1990; Griffin, 1993). However, *S. scapterisci* survived better at 10 and 25 °C than at 4 °C (Grewal *et al.*, 1993) and the heat tolerant *H. bacteriophora* IS5 stored better at room temperature (25 °C) than at 10 °C (Shapiro *et al.*, 1996).

Survival of naturally-occurring or artificially-applied entomopathogenic nematodes is necessary for the effective control of both soil-inhabiting and cryptic insects (Klein, 1990; Begley, 1990), but their survival is affected by the ambient temperature (Glazer, 1996; Kaya, 1990). For example, the survival of *S. carpocapsae* in soil declined as the temperature increased above 25 °C, whereas survival of *S. glaseri* was higher over the temperature range 10- 35 °C (Molyneux, 1985; Kung *et al.*, 1991). Temperatures above 35 °C may be lethal to the infective juveniles of species of *Steinernema* (Schmiege, 1963).

The capacity of entomopathogenic nematodes to withstand subzero temperatures has been investigated by several authors. *Steinernema anomali*, *S. feltiae* and *Heterorhabditis bacteriophora* survive using a freeze-tolerant strategy (Brown and

Gaugler, 1996), whereas *Heterorhabditis zealandica* use a freeze-avoiding strategy (Wharton and Surrey, 1994). For the freezing tolerant *S. feltiae*, *H. bacteriophora* and *S. anomali*, the lower lethal temperatures limits were -22, -19 and -14 °C, respectively (Brown and Gaugler, 1996). The sheath of infective juveniles of *H. zealandica* confers protection against freezing. Within the sheath, the pseudocoelomic fluid supercools up to -32 °C (Wharton and Surrey, 1994). In addition, several researchers have demonstrated that entomopathogenic nematodes could survive in liquid nitrogen at -195 °C (Curran *et al.*, 1992; Nugent *et al.*, 1996).

Temperature ranges for infectivity of entomopathogenic nematodes vary considerably (Grewal *et al.*, 1994; Mason and Hominick; 1995; Molyneux, 1986). Steinernematids are infective within a temperature range from 10 to 39 °C, with an optimum temperature range between 23 to 28 °C (Grewal *et al.*, 1994; Henneberry *et al.*, 1996; Molyneux, 1986; Williams and Macdonald, 1995). Heterorhabditids are infective within a temperature range from 7 to 35 °C, with an optimum temperature of 25 °C (Grewal *et al.*, 1994; Mason and Hominick; 1995; Molyneux, 1986).

The development and reproduction of entomopathogenic nematodes is influenced by temperature. The highest temperature (37.5 °C) capable of sustaining nematode development and reproduction was recorded for *Steinernema scapterisci* (Grewal *et al.*, 1993). Wright (1992) reported that only *S. feltiae* was able to develop at 10 °C, and that *S. carpocapsae*, *H. bacteriophora*, *H. zealandica* and *H. megidis* failed to develop at such a low temperature. No reproduction was observed in *H. heliothidis* at 5, 10 or 30 °C and

in *S. glaseri* at 5 or 10 °C (Zervos *et al.*, 1991). Similarly, no reproduction was noticed in *H. bacteriophora*, *H. zealandica*, *Heterorhabditis* sp Trinidad strain and *H. megidis* at 10 °C and in *H. megidis* UK strain and *H. zealandica* at 30 °C (Mason and Hominick, 1995).

There is some evidence to suggest that activity, survival, infectivity, development and reproduction may be modified by holding or propagating entomopathogenic nematodes in the laboratory at specified temperatures. (Brown and Gaugler, 1996; Grewal *et al.*, 1996; Griffin, 1996; Selvan *et al.*, 1996; Shapiro *et al.*, 1996). With respect to tolerance to freezing temperatures, it has been observed that *S. feltiae* and *H. bacteriophora* increased their capacities for freezing tolerance after acclimation to temperatures below which they were propagated under laboratory conditions (= recycling temperatures) (Brown and Gaugler, 1996). However, Surrey (1996) reported that the acclimation temperature did not increase the freezing survival in *H. zealandica*. In addition, the rearing method also influenced the capacities for temperature tolerance in these nematodes. Infective juveniles of *H. zealandica* survived better following freezing when reared in the insect host than when reared on an artificial medium (Surrey, 1996).

With respect to tolerance to high temperatures, Shapiro *et al.* (1996) reported that *H. bacteriophora* IS5 strain, isolated from the Negev desert in Israel then recycled at 30 °C, showed greater survival capacity than those recycled at 25 °C, whereas no such capacity was displayed by the HP88 strain of the same species. However, Selvan *et al.* (1996) demonstrated that the heat tolerance of *H. bacteriophora* HP88 strain to a

temperature (40 °C) above the recycling temperature was enhanced by pre-conditioning infective juveniles to 35 °C for a short time (1-3 hours).

Recycling and storage/acclimation temperatures also affect the infectivity, development and reproduction of entomopathogenic nematodes. The temperature limits for infectivity of *H. bacteriophora* and *S. anomali*, as well as for reproduction of the former species, were extended following prolonged subculturing at temperatures different from the stock cultures (Grewal *et al.*, 1996). Similarly, the infectivity and reproduction of *H. bacteriophora* IS5 strain were enhanced by recycling the nematodes at 30 °C rather than 25 °C (Shapiro *et al.*, 1996). However, the capacities for infectivity and reproduction were unaffected in *H. bacteriophora* HP88 strain, *H. heliothidis* and two strains of *S. feltiae* by propagating them at various temperatures (Dunphy and Webster, 1986; Shapiro *et al.*, 1996).

In addition, the prolonged storage (2-4 months) of *Heterorhabditis* sp at a cold (9 °C) temperature resulted in improved infectivity at the same temperature, possibly due to cold temperature- induced physiological acclimation (Griffin, 1996). Selvan *et al.* (1996) also showed that the infectivity of *H. bacteriophora* HP88 to temperature regimes above the recycling temperature was enhanced by pre-conditioning the infective juveniles to 35 °C for 1-3 hours time.

Thus, while it would appear that entomopathogenic nematodes infect, kill and reproduce within insects according to temperature profiles characteristic of their original habitats (Shapiro *et al.*, 1996; Grewal *et al.*, 1994; Grewal *et al.*, 1996; Molyneux, 1986),

such capacities are amenable to modification through laboratory manipulation.

1.3. Statement of research problem and objectives

From the foregoing, it is clear that the selected nematodes for pest management must be able to tolerate and adapt to temperatures prevailing within the biotopes of the insect pest(s). Ideally, the nematode to be deployed should have been acquired from an environment with a similar temperature profile to that of the targeted area to best ensure temperature compatibility (Griffin, 1993). However, since the biocontrol agent would have to be intensively and successively cultivated, it is important to determine the degree to which the temperature regime at which it is cultivated may affect tolerance to temperature extremes and infectivity, since these are among a plethora of factors that influence field efficacy.

Along with information on the effects of cultivation conditions on the nematodes at the whole organism level, it is also important to examine the underlying physiological mechanisms that enable the nematodes to adapt to the recycling temperatures and to temperature changes within their environment. The physiology of temperature adaptation in nematodes is poorly understood and additional information is needed to develop entomopathogenic nematodes as biocontrol agents.

This study examines the effects of various recycling temperatures on the thermal tolerances and infectivities of the Newfoundland (NF) strain of *S. feltiae* and four other strains of entomopathogenic nematodes and investigates the physiological mechanisms for

temperature adaptation and tolerance in three species of *Steinernema* recycled at various temperature regimes for a prolonged period (two years). The numbers of generations perpetuated at the various temperatures were estimated to be 15-18 (10 °C), 21-25 (15 °C), 30-35 (20 °C) and 48-60 (25 °C).

The specific objectives of this study were as follows:

I. Whole organism

(a) To identify an entomopathogenic nematode isolated from Newfoundland soil using classical morphological and biochemical methods.

(b) To study the effect of different recycling temperatures on the capacities for cold and warm temperature tolerance in the entomopathogenic nematode isolated in Newfoundland in comparison with species of entomopathogenic nematodes obtained from commercial suppliers and other researchers.

(c) To study the effect of various recycling temperatures on the infectivity of steinernematid nematodes at various bioassay temperatures.

II. Physiology

(a) To study the effect of various temperatures on the synthesis of isoenzymes of four metabolic enzymes in *Steinernema* species.

(b) To study the effect of recycling temperatures on the activities and kinetic properties of two key metabolic enzymes of entomopathogenic nematodes.

(c) To study the effect of temperatures on the proportion of saturated and unsaturated fatty acids in lipids of steinernematid nematodes.

CHAPTER 2

USE OF CELLULOSE ACETATE ELECTROPHORESIS IN THE TAXONOMY OF STEINERNEMATIDS (RHABDITIDA, NEMATODA)

2.1. ABSTRACT

A steinernematid nematode was isolated from soil samples collected near St. John's, Newfoundland, Canada and on the basis of its morphometry and Restriction fragment length polymorphisms (RFLP's) in ribosomal DNA spacer, it was designated as a new strain, NF, of *Steinernema feltiae*. Cellulose acetate electrophoresis was used to separate isozymes of eight enzymes in infective juveniles of *S. feltiae* NF strain as well as four other isolates: *S. feltiae* Umeå strain, *S. feltiae* L1C strain, *Steinernema carpocapsae* All strain and *Steinernema riobravis* TX strain. Based on comparisons of the relative electrophoretic mobilities (μ) of the isozymes, one of the eight enzymes (arginine kinase) yielded zymograms that were distinctive for each of the isolates, except for the Umeå and NF strains of *S. feltiae*, which had identical banding patterns. Four enzymes (fumarate hydratase; phosphoglucoisomerase, phosphoglucomutase, 6-phosphogluconate dehydrogenase) yielded isozyme banding patterns that were characteristic for all isolates, except for the L1C and NF strains of *S. feltiae*, which were identical. Two enzymes (aspartate amino transferase and glycerol-3-phosphate dehydrogenase) yielded zymograms that permitted *S. carpocapsae* All strain to be discriminated from the other four isolates, while the remaining enzyme (mannose-6-phosphate isomerase) was discriminatory for *S. riobravis* TX strain. Except for one enzyme, the isozyme banding pattern of the NF isolate of *S. feltiae* was the same as in the L1C strain, isolated 14 years ago from Newfoundland. Cellulose acetate electrophoresis could prove invaluable for taxonomic identification of isolates of steinernematids, provided that a combination of enzymes is used.

2.2. INTRODUCTION

Nematodes of the genus *Steinernema* have attracted considerable attention for development as novel biopesticides. Infective juveniles of these nematodes gain access to the hemocoel of soil-inhabiting insect hosts via natural openings (Poinar, 1990), then release a mutualistic bacterium which kills the host and creates a nutritional milieu conducive for nematode development.

There are currently 18 recognized species of *Steinernema* (Akhurst, 1995), several of which exist as geographically distinct strains (Poinar, 1990). The ability to be able to identify strains is an important aspect of the overall research and development of this family of entomopathogenic nematodes as biocontrol agents. Curran (1990) concluded that DNA sequence analysis constitutes the preferred molecular method for discriminating between categories of entomopathogenic nematodes below the species level. To facilitate this, DNA amplification techniques by polymerase chain reaction (PCR) have been developed for entomopathogenic nematodes (Liu and Berry, 1995).

Protein electrophoresis also could be a useful taxonomic tool, enabling nematologists to more rapidly and cost effectively discriminate between species (Curran and Webster, 1984; Sherman and Jackson, 1963), and in some instances, between strains (Huettel *et al.*, 1983). With respect to entomopathogenic nematodes, starch gel electrophoresis revealed an array of esterase and alkaline phosphatase isozymes in *Steinernema glaseri* that was qualitatively and quantitatively different from that in *S. carpocapsae* (Sherman and Jackson, 1963). This same procedure, adapted to separate

isozymes of several enzymes, was used by Akhurst (1987) to distinguish between 22 strains representing 3-8 species of *Heterorhabditis*. Polyacrylamide gel electrophoresis was used to discriminate between five isolates of *Steinernema* on the basis of their esterase isozyme patterns (Sha, 1985) and ten isolates belonging to four species of this genus according to their total protein, nonspecific esterase and alkaline phosphatase patterns (Kozodoi *et al.*, 1986). Poinar and Kozodoi (1988) showed that two morphologically similar steinernematids, *S. glaseri* and *Steinernema anomali*, possessed dissimilar and distinctive enzyme patterns, as revealed by polyacrylamide gel electrophoresis.

Cellulose acetate is a simpler, more rapid type of electrophoresis, regarded by some authorities as being more sensitive and providing superior resolution to starch gel or polyacrylamide gel electrophoresis (Easteal and Boussy, 1987). It has not been used in the taxonomy of entomopathogenic nematodes.

This chapter reports upon the identification of a steinernematid, *S. feltiae* NF strain, isolated from soil in Newfoundland. This identification process includes an evaluation of the taxonomic value of cellulose acetate electrophoresis by separating isozymes of eight metabolic enzymes in five isolates of *Steinernema*: *S. carpocapsae* All strain, *S. riobravis* TX strain, *S. feltiae* Umeå strain (= S. *carpocapsae* Umeå strain), *S. feltiae* L1C strain and *S. feltiae* NF strain.

2.3. MATERIALS AND METHODS

2.3.1. Sources of nematodes

Steinernema carpocapsae All strain was provided by Plant Products Ltd., Brampton. Ontario. Canada; *S. riobravis* TX strain by Dr. H. E. Cabanillas, USDA, ARS, Crop Insects Research Unit, Weslaco, TX. *S. feltiae* Umeå strain was provided by Dr. R. West. Canadian Forest Service (CFS), St. John's. NF, Canada from a stock colony that had been initially obtained from Biologic Biocontrol Products, Willow Hill, PA. *Steinernema feltiae* L1C strain was obtained from a colony being maintained at CFS, St. John's, NF. This nematode was isolated in 1983 (Finney-Crawley, 1985) from a location close to St. John's. NF and has been maintained in laboratory culture at CFS since then. The original collection site has been paved over. *S. feltiae* NF strain is a new strain that I isolated in Summer 1993 (Jagdale *et al.*, 1996) from soil on an organic garden close to St. John's, NF. using *Galleria* bait traps (Woodring and Kaya, 1988). All nematode isolates were recycled once by propagation through *G. mellonella* larvae (Woodring and Kaya, 1988; see Appendix I) at 25 °C before being subjected to various recycling regimes.

2.3.2. Identification of the Newfoundland (NF) isolate

This was accomplished by measurements of the infective third-stage juvenile and by restriction fragment length polymorphisms of amplified rDNA. Infective juveniles of *S. feltiae* NF strain were collected from White traps (Woodring and Kaya, 1988)

immediately after emergence from the *Galleria* cadavers i.e.. 7-10 days after infection. Nematodes were heat killed by placing them in a small beaker containing water (room temperature), which was then plunged into another beaker filled with water (100 °C) for 2-3 minutes or until the specimens assumed the almost straight form characteristic of heat death (Hooper, 1986). The killed nematodes were fixed and stained for 24 hours in Chang's nerve stain (Gray, 1975), then differentiated repeatedly in warm lactophenol (40-50 °C) until no more superfluous stain was evident, then mounted in glycerin (Hooper, 1986). Measurements of 25 randomly selected infective juveniles were made under a light microscope (X 1,000) equipped with a micrometer eyepiece. The morphological parameters and ratios measured were those used by Poinar (1990).

Molecular taxonomy via rDNA analysis was carried out by Dr. T. C. Vrain, Agriculture and Agri-Food Canada, Summerland, BC, Canada. Restriction fragment length polymorphisms of amplified rDNA using internally transcribed spacer (ITS) fragments were used to separate the NF strain from other commercially used strains of *Steinernema* (*S. carpocapsae* All strain, or *S. feltiae* Umeå) and the L1C strain. Other strains of *S. feltiae* type A (Sf76, Sf509a, Sf519) or *S. feltiae* type B (B1 Nashes and B2 216) were provided by Dr. Alex Reid (Imperial College of Science, Technology and Medicine, Department of Biology, Berks, UK). The strains of *S. carpocapsae* (DD136, 42, Breton, Kapow) were provided by Dr. Martin Hubbes (Forestry Department, University of Toronto, Ontario, Canada). Nematodes were stored at -20 °C until use. For each population, five nematodes were placed in a 5 µl drop of lysis buffer (1 ml of lysis buffer

contained 100 µl 10% SDS [sodium dodecyl sulfate], 100 µl M Tris [Tris (hdroxymethyl) aminomethane] pH 8.7, 40 µl 5 M NaCl, 10 µl 0.5 M EDTA [Ethylenediaminetetraacetic acid], 750 µl deionized water) on a silated glass cover slip, and cut open with a piece of razor blade. The resulting suspension was pipetted into a 1.5 ml microfuge tube and incubated at 60 °C for 1 hour following the addition of 15 µl of lysis buffer with 40 µg of proteinase K (Sigma Chemical Co. St. Louis, MO.). DNA was recovered from the suspension by binding to powdered glass (Geneclean kit, Bio/Can Scientific, Mississauga, ON. Canada), and eluted into 10 µl deionized water. Amplification of ribosomal ITS sequences using the PCR was as described in Vrain et al. (1992). Two primers, 21 base pairs long, one with sequence at the 3´ end of the 18 S gene and the other at the 5´ end of the 26 S gene, allowed for the amplification of a rDNA fragment composed of the 3´ end of the 18 S gene, the ITS 1, the 5.8 S gene, the ITS 2, and the 5´ end of the 26 S gene. Negative controls containing all necessary chemicals but without added DNA, and positive controls containing *Caenorhabditis elegans* DNA, were included in all experiments. The amplified ribosomal ITS fragments were digested using five restriction enzymes, Eco RI, Hae III, Hinf I, Mbo I, and Rsa I, in a 10 µl volume according to manufacturer's instructions (Pharmacia, Uppsala, Sweden). Fragments of each restriction digest were separated by electrophoresis and visualized in the agarose gels (Vrain *et al.*, 1992).

2.3.3. Cellulose Acetate Electrophoresis Studies

2.3.3.1. Enzyme extraction

Infective juveniles of each of the five isolates were transferred from the dilute formalin in the White traps (Woodring and Kaya, 1988) to separate 250 ml beakers containing distilled water, then allowed to settle (ca. 10 min). Using an Eppendorf micropipeter, infective juveniles were transferred from the bottom of the beakers to Whatman no. 4 filter papers, so that they formed confined blobs on the papers. Samples of each isolate (10 mg wet weight) were transferred into separate polypropylene microcentrifuge tubes (1.5 ml), then macerated in the tubes with a pellet pestle mixture (Baxter Diagnostic Corporation, Canlab Division, Mississauga, ON, Canada). After maceration, 30 µl of distilled water containing bromophenol blue tracking dye (pH 6.0) was added to each tube, the homogenates centrifuged at 3,200g for 2 minutes, then held on ice. Aliquots (10 µl) of the supernatants were transferred from the tubes to separate wells in the sample holder and kept on ice until the loading of the samples was performed on the cellulose acetate plates (Helena Laboratories, Beaumont, TX).

2.3.3.2. Cellulose acetate electrophoresis procedure

Aliquots (0.6 µl) of the supernatants were transferred from the sample holder to Titan III Zip Zone Cellulose Acetate Plates using a Super Z Applicator (Helena Laboratories). Plates had been pre-soaked (20 min) in Tris-Glycine buffer (3.0 g Tris, 14.4 g glycine, 1L distilled water, pH 8.5) prior to spotting. Electrophoresis was carried out (2

mA/ plate: 20-30 min) in Tris-Glycine buffer in a horizontal electrophoresis chamber at room temperature (20-25 °C).

2.3.3.3. Enzyme staining

Specific stains were used to visualise the following enzymes: arginine kinase (EC 2.7.3.3), aspartate amino transferase (EC 2.6.1.1), fumarate hydratase (EC 4.2.1.2), glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), mannose-6-phosphate isomerase (EC 5.3.1.8), phosphoglucoisomerase (EC 5.3.1.1), phosphoglucomutase (EC 2.7.5.1) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) (Hebert and Beaton, 1993).

Plates were removed from the electrophoresis chamber and placed on a levelled glass surface. Melted agar (60 °C) was added to the stain mixture, then the resultant solution poured immediately over the plates, which were then incubated in the dark until isoenzyme bands became prominent (ca. 10 - 15 min). Plates were then washed (2-3 times) under tap water and the bands fixed by immersion in acetic acid : methanol : distilled water (1:4:10) for 10 minutes. Fixed plates were dried over-night, then photographed and isozyme bands measured. Three replicate plates were prepared for each nematode isolate, each replicate representing a separate homogenate of infective juveniles. The relative electrophoretic mobility (μ) for each isoenzyme was measured to compare the migration rates (Lehninger, 1979). Isozyme bands among isolates were considered the same if their μ values were within 10% of one another. This margin of error was selected because the highest and lowest μ values among three replicates of the same isozyme were

always found to be within 10% of one another.

2.4. RESULTS

2.4.1. Identification of the Newfoundland (NF) isolate

The measurements and ratios of morphometric characters of the Newfoundland isolate corresponded with those reported for *S. feltiae* (Table 1). Restriction fragment length polymorphisms separated the NF strain from *S. carpocapsae* All and Umeå strains (Table 2). The Umeå strain was clearly associated with the restriction fragment length polymorphism type A of *S. feltiae* as defined by Reid and Hominick (1992), while the *S. feltiae* NF strain appeared to belong to the restriction fragment length polymorphism type B (Reid and Hominick, 1992). No polymorphisms were identified which distinguished between *S. feltiae* L1C and NF strains using five restriction enzymes to digest the rDNA ITS amplified fragment. Thus, on the basis of morphometry and RFLP's in ribosomal DNA ITS, the isolate is designated as *S. feltiae* NF strain.

2.4.2. Cellulose Acetate Electrophoresis Studies

2.4.2.1. Arginine kinase (ARK)

The isozyme banding pattern for this enzyme was strain specific. Each of the five isolates possessed a single band of ARK activity that migrated anodally (Fig. 1A).

According to measurements of electrophoretic mobility (Table 3), the same anodal band (Band -2) was common to the three strains of *S. feltiae*. *Steinernema carpocapsae* All strain and *S. riobravis* TX strain possessed a different anodal band (Band -1). The three strains of *S. feltiae* and *S. riobravis* TX strain possessed a common cathodal band of ARK activity (Band 1) with the same electrophoretic mobility (Table 3). *Steinernema feltiae* L1C strain possessed a second cathodal band (Band 2), while *S. carpocapsae* All strain possessed two cathodal bands (Bands 3,4) that were electrophoretically distinct from those in any of the other four isolates.

2.4.2.3. Aspartate amino transferase (AAT)

The isozyme banding patterns (AAT) of *S. feltiae* Umeå strain and *S. riobravis* TX strain were identical, as were those for the L1C and NF strains of *S. feltiae*. *Steinernema carpocapsae* All strain had a distinct banding pattern (Fig. 1B). There was a common anodal band (Band -1) in *S. carpocapsae* All strain, *S. feltiae* Umeå strain and *S. riobravis* TX strain, but the L1C and NF strains of *S. feltiae* possessed no such band (Table 3). Four of the isolates possessed one common cathodal band (Band 1), whereas *S. carpocapsae* All strain had a band (Band 2) that migrated more rapidly toward the cathode (Fig. 1B). The banding pattern of the Umeå strain of *S. feltiae* was distinct from the other two strains (L1C and NF) of the same species due to the fact that it possessed an additional anodal band (Band -1).

2.4.2.2. Fumarate hydratase (FUM)

Only the L1C and NF strains of *S. feltiae* had identical zymograms, consisting of three bands (Bands 1, 6, 7) that migrated toward the cathode (Fig. 1C). The Umeå strain of *S. feltiae* also possessed three cathodal bands, the slowest moving of which (Band 1) was common to all strains of this species. *Steinernema carpocapsae* All strain possessed two cathodal bands, including the slowest moving band present in *S. feltiae*. There were four discernible bands in *S. riobravis* TX strain, one of which (Band 3) was present in *S. carpocapsae* All strain and two of which (Bands 4, 5) were present in *S. feltiae* Umeå strain (Table 3).

2.4.2.4. Glycerol-3-phosphate dehydrogenase (GPDH)

Only *S. carpocapsae* All strain had a GPDH banding pattern that was distinguishable from the other isolates. All isolates possessed a single band of activity that migrated toward the cathode (Fig. 1D). The electrophoretic mobility (Table 3) was greater in *S. carpocapsae* All strain than in the other four isolates, which shared a common isozyme (Fig. 1D).

2.4.2.5. Mannose-6-phosphate isomerase (MPI)

Isozymes of this enzyme were not strain or species specific. Each of the isolates possessed two bands of MPI activity that migrated toward the cathode (Fig. 2A). According to μ values (Table 3), these two bands (Bands 3,4) were identical in the NF

and L1C strains of *S. feltiae*. The Umeå strain of *S. feltiae* had the same two isozyme bands (Bands 1,2) as the All strain of *S. carpocapsae*. One of the two isozyme bands (Band 4) in *S. riobravis* TX strain was also present in *S. feltiae* L1C and NF strains. However, *S. riobravis* TX strain had a distinctive isozyme pattern (Fig. 2A).

2.4.2.6. Phosphoglucoisomerase (PGI)

Only the L1C and NF strains of *S. feltiae* had identical isozyme profiles (Fig. 2B). *Steinernema riobravis* TX strain possessed one cathodal band; the remaining four isolates possessed two bands (Fig. 2B). One of the bands (Band 4) was common to all three strains of *S. feltiae* (Table 3).

2.4.2.7. Phosphoglucomutase (PGM)

With the exception of the L1C and NF strains of *S. feltiae*, isolates possessed distinct zymograms (Fig. 2C). There were two cathodal bands (Bands 2, 4) common to *S. feltiae* L1C and NF strains. *Steinernema riobravis* TX strain also possessed two bands, one of which (Band 4) had the same as *S. feltiae* NF and L1C strains (Table 3). *Steinernema carpocapsae* All strain possessed three, *S. feltiae* Umeå strain four cathodal bands; two of these bands (Bands 3,5) were common to both species (Fig. 2C; Table 3).

2.4.2.8. 6-Phosphogluconate dehydrogenase (6PGDH)

Only the L1C and NF strains of *S. feltiae* had identical zymograms (Fig. 2D),

consisting of two cathodal bands (Bands 1,2). *Steinernema feltiae* Umeå strain possessed two bands (Bands 3,4) with values different from those in the other two *S. feltiae* strains (Table 3). *Steinernema carpocapsae* All strain and *S. riobravis* TX strain each possessed one cathodal band which, in the case of *S. riobravis* TX strain, was the same as was present in *S. feltiae* Umeå strain (Fig. 2D).

2.5. DISCUSSION

Cellulose acetate electrophoresis proved effective in discriminating between the five isolates of *Steinernema*. Results reported in this study have been replicated on several occasions. One of the eight enzymes studied (ARK) yielded zymograms that were distinctive for each of the isolates, except for the Umeå and NF strains of *S. feltiae*, which had identical banding patterns. Four enzymes (FUM; PGI; PGM; 6 PGDH) had isozyme profiles that were characteristic for all isolates except for the LIC and NF strains of *S. feltiae*, which were indistinguishable. Three enzymes (AAT; GPDH; MPI) provided banding patterns that did not permit strain or species separations to be made. In the case of AAT and GPDH, only *S. carpocapsae* All strain had a distinguishing banding pattern, while *S. riobravis* TX strain was the only such isolate with respect to MPI zymograms. Thus, while no single enzyme would be sufficient to allow taxonomic identification among the five isolates, a combination of enzymes would permit such a task.

The steinernematid nematode isolated from soil samples in Newfoundland may,

on the basis of its morphometry and RFLP's in ribosomal ITS be regarded as a boreally adapted strain of *S. feltiae*. The fact that the L1C and NF strains of *S. feltiae* had identical isozyme profiles for seven of the eight enzymes is understandable, since these two strains were isolated from soil samples taken approximately 10 km from one another. Since the ribosomal DNA profiles of LIC and NF strains are indistinguishable, they had initially considered to be the same strain. However, *S. feltiae* LIC strain was isolated in 1983 (Finney-Crawley, 1985) and has been maintained in laboratory culture since then. It is possible that it may have undergone genetic and consequent biochemical changes resulting from repetitive laboratory recycling and that the loss of one of the cathodal bands of ARK activity constitutes one manifestation of such transformation. The possibility that the two closely related strains may have always been distinct from the time of collection cannot be investigated, because the original collection site for the L1C strain has been paved over and it was determined that soil from adjacent areas is free of entomopathogenic nematodes.

Based on its morphometry (Jagdale and Gordon; unpubl. obs.), isozymic patterns of six enzymes (AAT, FUM, MPI, PGI, PGM and 6PGDH), and RFLP's in the ribosomal ITS spacer as reported herein, the nematode first isolated from soil in Sweden (Pye and Pye, 1985) and since marketed commercially as *S. carpocapsae* Umeå strain should be reclassified as *S. feltiae*. This designation further accords with studies done on its bacterial symbiont. On the basis of biochemical and physiological characters, Boemare and Akhurst (1988) designated the bacterial symbiote of the Umeå isolate as *Xenorhabdus bovienii*, a

bacterial species normally associated with *S. feltiae*. The overall isozyme profiles of this nematode contained elements of all three species. For example, it had a zymogram for ARK identical to *S. feltiae* NF strain, lacking all three isozymes present in *S. carpocapsae* All strain. However, its MPI zymogram was identical only to *S. carpocapsae* All strain, and its GPDH zymogram consisted of the same single isozyme produced by *S. feltiae* and *S. riobravis*, but not by *S. carpocapsae*. However, the Umeå strain was readily distinguishable from the other two strains of *S. feltiae*, NF and L1C, on the basis of its isozyme banding patterns.

Electrophoretic procedures, starch gel (Akhurst, 1987; Huettel *et al.*, 1983), polyacrylamide gel electrophoresis (Ibrahim *et al.*, 1994; Paggi *et al.*, 1991) and isoelectric focusing (Fukumoto *et al.*, 1988; Payan and Dickson, 1990) have been widely deployed for taxonomic separations of species and subordinate taxa of animal parasitic and plant parasitic nematodes. Cellulose acetate electrophoresis is a relatively inexpensive procedure that has the added benefit of speed. Pre-run times are reduced to a minimum by utilizing pre-manufactured plates and running times are as low as 20-30 minutes. Based on the experience of this study, it affords the necessary sensitivity to allow good resolution of isozymes from small amounts of nematode material. The current interest in commercializing steinernematids has resulted in a burgeoning inventory of isolates from many parts of the world (Poinar, 1990). While based on only three species that embody five strains, the present study nevertheless suggests that cellulose acetate electrophoresis could be of practical use to researchers, enabling species and even strain designations of

isolates to be made. More extensive studies are warranted, to provide for the cataloging of a broad array of isolates and enzymes.

Table 1. Morphometric characters (in µm) of infective juveniles of *Steinernema feltiae* NF strain[a]

Character/ Ratio	*S. feltiae* NF strain	*S. feltiae*[b]
Body length (L)	857.6 ± 65.2 (675.0-937.5)	849 (736-950)
Greatest width (W)	35.50 ± 5.72 (25.0-45.0)	26 (22-29)
Distance from head to excretory pore (EP)	66.6 ± 9.4 (55.0-87.5)	62 (53-67)
Distance from head to nerve ring (NR)	92.3 ± 11.5 (80.0-117.5)	99 (88-112)
Distance from head to pharynx base (PB)	131.0 ± 14.9 (105.0-162.5)	136 (115-150)
Tail length (T)	81.3 ± 8.5 (62.5-100)	81 (70-92)
Ratio A (L/W)	24.7 ± 4.0 (18.0-34.0)	31 (29-33)
Ratio B (L/PB)	6.6 ± 0.8 (5.3-8.0)	6 (5.3-6.4)
Ratio C (L/T)	10.6 ± 1.0 (9.0-13.2)	10.4 (9.2-12.6)
Ratio D (EP/PB)	0.5 ± 0.05 (0.42-0.61)	0.45 (0.42-0.51)
Ratio E (EP/T)	0.81 ± 0.11 (0.64-1.11)	0.78 (0.69-0.86)

[a] Mean values ± SD (n= 25). Ranges are given in parentheses.
[b]From Poinar (1990).

Table 2. Size of DNA fragments generated by restriction of PCR amplified ribosomal sequence in strains of *S. feltiae* and *S. carpocapsae*.

Species	Strains	Restriction Enzymes		
		Hinf I	Mbo I	Rsa I
S. carpocapsae	All, DD136, 42, Breton, Kapow	420 300 280 150	590 240 190	400 380 320
S. feltiae type A	Sf76, Sf509a, Sf519	720 240 180	590 240 120 100	460 240 220 140
S. feltiae type B	B1 Nashes, B2 216	720 240 160	340 300 190 120 100	720 220 140
S. feltiae	Umeå	720 240 180	590 240 120 100	460 240 220
	NF	720 240 160	340 300 190 120 100	720 220 140
	L1C	720 240 160	340 300 190 120 100	720 220 140

Size of DNA fragments in bp. Fragments smaller than 100 bp could not be measured accurately and are not included. Molecular taxonomy via rDNA analysis was carried out by Dr. T. C. Vrain, Agriculture and Agri-Food Canada, Summerland, BC, Canada.

Fig. 1. Photographs of comparative isoenzyme patterns for four enzymes exhibited by five nematode isolates. All= *Steinernema carpocapsae* All strain, U= *S. feltiae* Umeå strain. L1C= *S. feltiae* L1C strain. NF= *S. feltiae* NF strain, TX = *S. riobravis* TX strain. A) Arginine kinase (ARK). B) Aspartate aminotransferase (AAT). C) Fumarate hydratase (FUM) and D) Glycerol-3-phosphate dehydrogenase (GPDH). Arrows indicate points of sample application. Bands were numbered cathodal or anodal (-) in increasing numerical order relative to the distance that they migrated away from the origin.

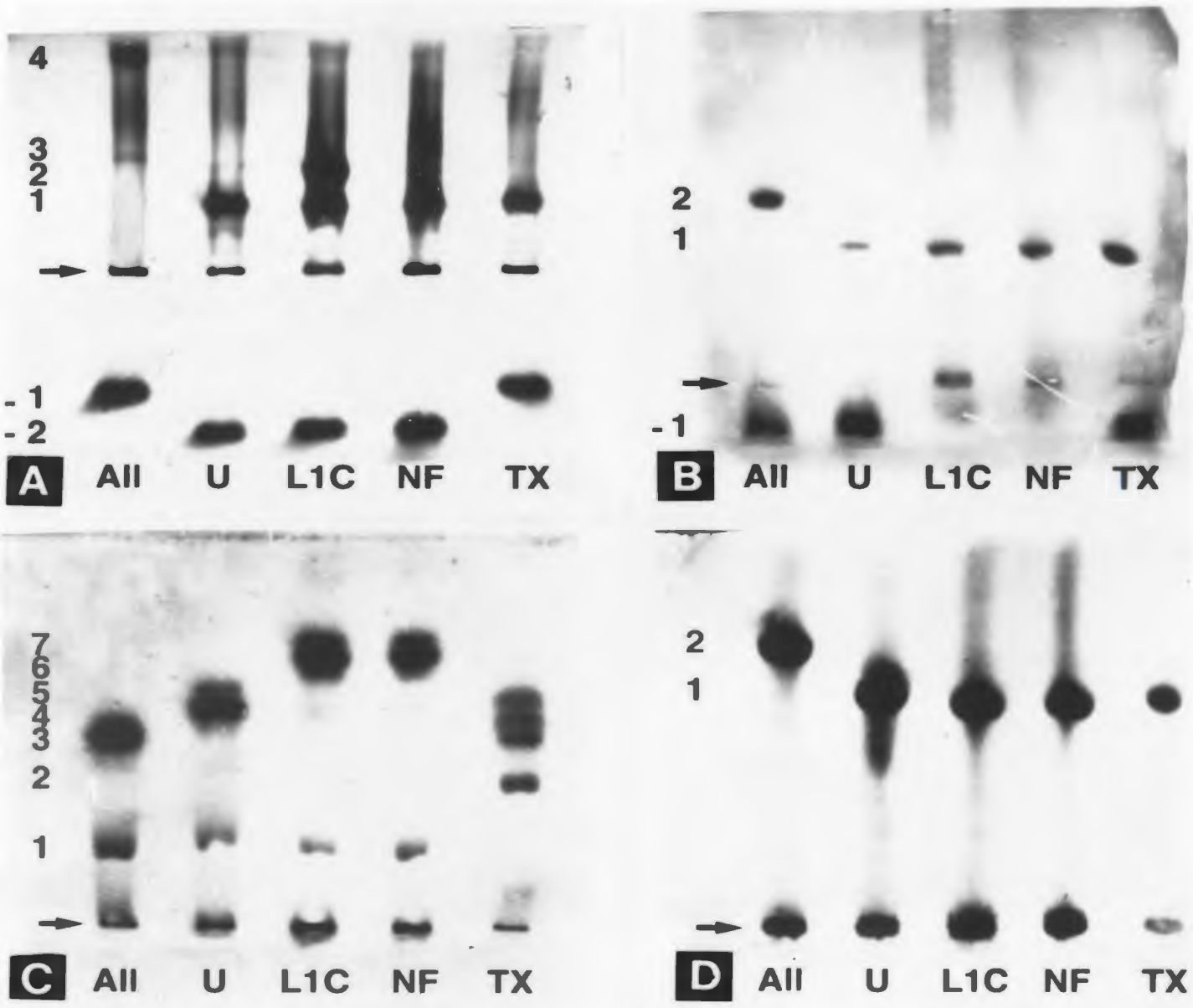
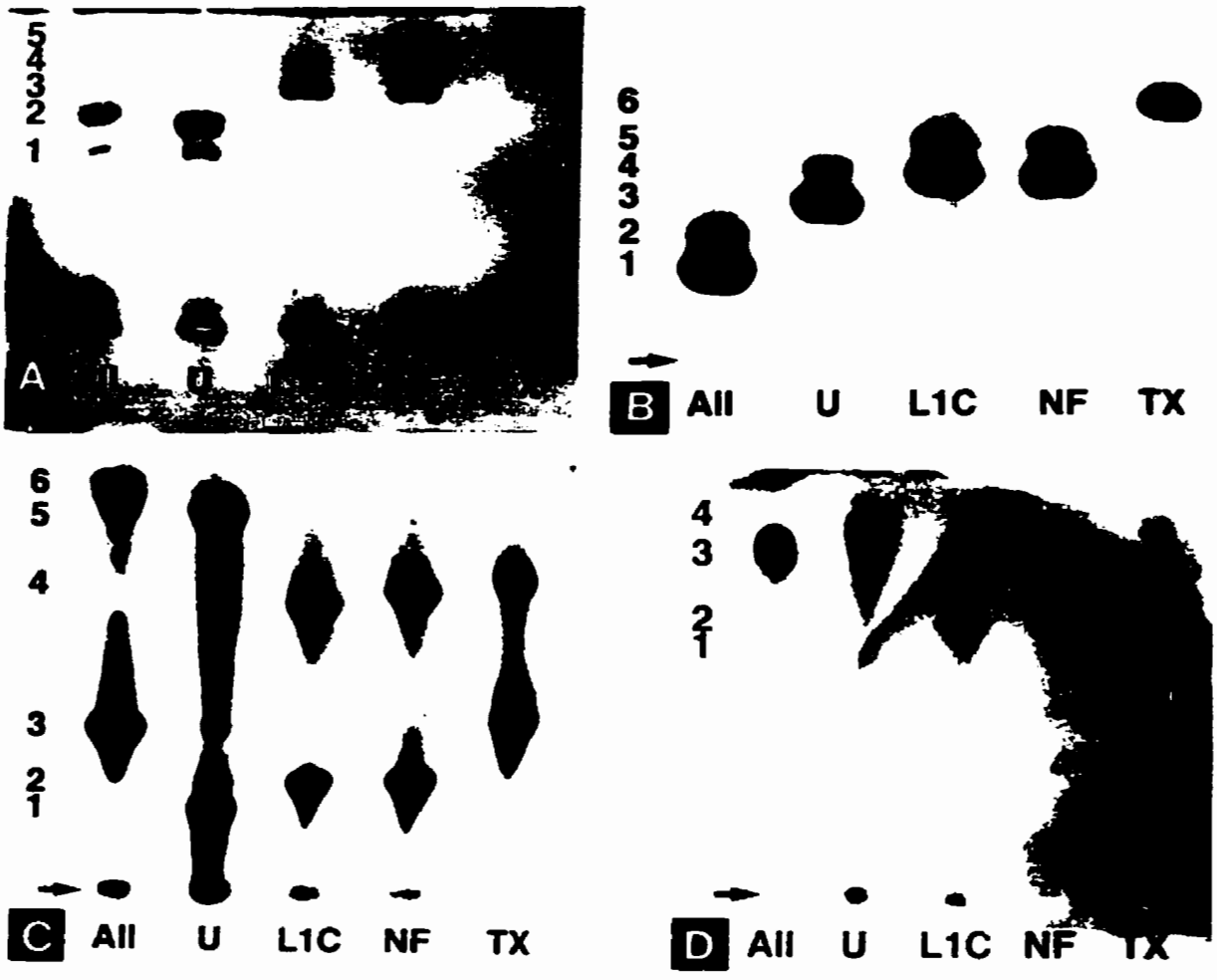

4
3
2
1
-1
-2
A
All
U
L1C
NF
TX
2
1
-1
B
All
U
L1C
NF
TX
7
6
5
4
3
2
1
C
All
U
L1C
NF
TX
2
1
D
All
U
L1C
NF
TX

Fig. 2. Photographs of comparative isoenzyme patterns for four enzymes exhibited by five nematode isolates. All= *Steinernema carpocapsae* All strain, U= *S. feltiae* Umeå strain, L1C= *S. feltiae* L1C strain, NF= *S. feltiae* NF strain, TX = *S. riobravis* TX strain. A) Mannose-6-phosphate isomerase (MPI), B) Phosphoglucoisomerase (PGI), C) Phosphoglucomutase (PGM) and D) 6-phosphogluconate dehydrogenase (6PGDH). Arrows indicate points of sample application. Bands were numbered cathodal or anodal (-) in increasing numerical order relative to the distance that they migrated away from the origin.


5
4
3
2
1
A
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6
5
4
3
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B
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L1C
NF
TX
6
5
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3
2
1
C
All
U
L1C
NF
TX
4
3
2
1
D
All
U
L1C
NF
TX

Table 3. Mean electrophoretic mobility (cm^2/sec./v) of isoenzymes of eight enzymes in five isolates of steinernematid nematodes.

# of[a] band	*S. c.* All[b]	*S. f.* Umeå	*S. f.* LIC	*S. f.* NF	*S. r.* TX
		Arginine kinase (ARK)			
(-) 1.	2.05×10^{-4}	-	-	-	1.99×10^{-4}
(-) 2.	-	2.69×10^{-4}	2.71×10^{-4}	2.71×10^{-4}	-
1.	-	1.10×10^{-4}	1.10×10^{-4}	1.10×10^{-4}	1.10×10^{-4}
2.	-	-	1.65×10^{-4}	-	-
3.	1.98×10^{-4}	-	-	-	-
4.	3.25×10^{-4}	-	-	-	-
		Aspartate amino transferase (AAT)			
(-) 1.	4.01×10^{-5}	3.78×10^{-5}	-	-	4.01×10^{-5}
1.	-	1.68×10^{-4}	1.54×10^{-4}	1.51×10^{-4}	1.60×10^{-4}
2.	2.07×10^{-4}	-	-	-	-
		Fumarate hydratase (FUM)			
1.	1.08×10^{-4}	1.16×10^{-4}	1.16×10^{-4}	1.08×10^{-4}	-
2.	-	-	-	-	1.55×10^{-4}
3.	2.05×10^{-4}	-	-	-	1.97×10^{-4}
4.	-	2.31×10^{-4}	-	-	2.31×10^{-4}
5.	-	2.54×10^{-4}	-	-	2.57×10^{-4}
6.	-	-	2.90×10^{-4}	2.89×10^{-4}	-
7.	-	-	3.15×10^{-4}	3.17×10^{-4}	-
		Glycerol-3-phosphate dehydrogenase (GPDH)			
1.	-	2.40×10^{-4}	2.40×10^{-4}	2.40×10^{-4}	2.48×10^{-4}
2.	2.98×10^{-4}	-	-	-	-

Table 3 continued

# of[a] bands	*S. c.* All[b]	*S. f.* Umeå	*S. f.* LIC	*S. f.* NF	*S. r.* TX
		Mannose-6-phosphate isomerase (MPI)			
1.	1.50×10^{-4}	1.48×10^{-4}	-	-	-
2.	1.80×10^{-4}	1.72×10^{-4}	-	-	-
3.	-	-	1.99×10^{-4}	1.93×10^{-4}	-
4.	-	-	2.25×10^{-4}	2.23×10^{-4}	2.17×10^{-4}
5.	-	-	-	-	2.36×10^{-4}
		Phosphoglucoisomerase (PGI)			
1.	1.67×10^{-4}	-	-	-	-
2.	2.04×10^{-4}	-	-	-	-
3.	-	2.21×10^{-4}	-	-	-
4.	-	2.48×10^{-4}	2.45×10^{-4}	2.48×10^{-4}	-
5.	-	-	2.72×10^{-4}	2.69×10^{-4}	-
6.	-	-	-	-	3.08×10^{-4}
		Phosphoglucomutase (PGM)			
1.	-	5.65×10^{-5}	-	-	-
2.	-	8.53×10^{-5}	8.29×10^{-5}	8.22×10^{-5}	-
3.	1.41×10^{-4}	1.47×10^{-4}	-	-	1.42×10^{-4}
4.	-	-	2.64×10^{-4}	2.80×10^{-4}	2.84×10^{-4}
5.	3.73×10^{-4}	3.70×10^{-4}	-	-	-
6.	3.92×10^{-4}	-	-	-	-

Table 3 continued

# of[a] bands	*S. c.* All[b]	*S. f.* Umeå	*S. f.* L1C	*S. f.* NF	*S. r.* TX
		6-Phosphogluconate dehydrogenase (6PGDH)			
1.	-	-	2.41×10^{-4}	2.41×10^{-4}	-
2.	-	-	2.65×10^{-4}	2.65×10^{-4}	-
3.	3.11×10^{-4}	2.99×10^{-4}	-	-	-
4.	-	3.54×10^{-4}	-	-	3.47×10^{-4}

Values are the means of three replicate plates, each replicate representing a separate homogenate of infective juveniles.

[a] Bands were numbered cathodal or anodal (-) in increasing numerical order relative to the distance that they migrated away from the origin. Bands among isolates were considered the same if their μ values were within 10% of one another. Dashes indicate absence of bands.

[b] *S. c.* = *Steinernema carpocapsae*, *S. f.* = *S. feltiae*, *S. r.* = *S. riobravis*. All, Umeå, L1C, NF and TX are strain designations.

CHAPTER 3

EFFECT OF RECYCLING TEMPERATURES ON TEMPERATURE TOLERANCES OF ENTOMOPATHOGENIC NEMATODES.

3.1. ABSTRACT

Two strains (NF and Umeå) of *Steinernema feltiae*, *Steinernema carpocapsae* All strain and *Steinernema riobravis* TX strain were recycled at 25, 20 and 15 and 10 °C (where possible) for 2 years, then their capacities for tolerating high temperatures and freezing were determined. In all isolates, the UT_{50}, the upper temperature at which 50% of the infective juveniles died, increased with higher recycling temperatures. Tolerance to freezing, as measured by times at which 50% of the infective juveniles were killed by a -5 °C regime, was diminished at higher maintenance temperatures. The infectivity of nematodes that survived freezing was ≥ 90% that of unfrozen nematodes (controls). Two strains of *S. feltiae* were the most cold tolerant of the four isolates, but their capacities to withstand freezing were diminished by recycling at warmer temperatures. *Steinernema riobravis*, with the highest UT_{50} values, was the most heat tolerant, while *S. carpocapsae* All strain displayed an intermediate degree of tolerance to high temperatures.

3.2. INTRODUCTION

The importance of temperature as a key environmental factor that affects the dispersal, infectivity, reproduction and development of entomopathogenic nematodes (f. Steinernematidae and Heterorhabditidae) is well established (Kaya, 1990; Grewal *et al.*, 1994; Mason and Hominick, 1995; Molyneux, 1985; Griffin, 1993; Steiner, 1996). Nevertheless, infective juveniles of entomopathogenic nematodes have been recovered from the soils of a wide variety of climatic regions (Amarashinghe *et al.*, 1994; Cabanillas

et al., 1994; Griffin *et al.* 1991; Jagdale *et al.*, 1996; Mràček and Webster, 1993). Such nematodes are able to survive at habitat temperatures that undergo daily and/or seasonal cycles of fluctuation. To compensate for potentially lethal temperatures, infective juveniles may move deep into the soil (Kaya, 1990) or extend their thermal tolerance limits through processes of acclimatization.

It is possible that the thermal tolerance of nematodes such as steinernematids may be amenable to adjustment through laboratory acclimation involving prolonged maintenance or recycling at defined temperatures. Dunphy and Webster (1986) concluded that *Heterorhabditis heliothidis* and two strains of *S. feltiae* had no such capacity for temperature adaptation resulting from subculturing the nematodes at various temperatures. However, after a more prolonged subculturing regime at temperatures different from the stock cultures, the temperature limits for virulence and establishment of *Heterorhabditis bacteriophora* and *Steinernema anomali*, as well as for reproduction of the former species, were extended (Grewal *et al.*, 1996). Heat tolerance, infectivity and reproduction of a strain of *H. bacteriophora* isolated from the Negev desert in Israel were enhanced by rearing the nematodes at a warmer temperature, whereas no such effects were noted for the commercially available HP88 strain of the same species (Shapiro *et al.*, 1996). The purpose of the present study was to investigate whether the heat and cold tolerances of three species of *Steinernema* were affected by the temperature regimes at which they were recycled.

3.3. MATERIALS AND METHODS

3.3.1. Sources of nematodes

Steinernema carpocapsae All strain was provided by Plant Products Ltd., Brampton. Ontario, Canada; *S. riobravis* TX strain by Dr. H. E. Cabanillas, USDA, ARS, Crop Insects Research Unit, Weslaco, TX., U.S.A. *S. feltiae* Umeå strain was provided by Dr. R. West. Canadian Forest Service (CFS), St. John's, NF, Canada from a stock colony that had been obtained initially from Biologic Biocontrol Products, Willow Hill, PA., U.S.A. *Steinernema feltiae* NF strain is a new strain (Jagdale *et al.*, 1996) that was isolated in Summer 1993 from soil on an organic garden close to St. John's, NF, using *Galleria* bait traps (Woodring and Kaya, 1988).

3.3.2. Recycling temperature regimes

All nematode strains were maintained for two years (May, 1994- May, 1996) by propagation through *G. mellonella* larvae (Woodring and Kaya, 1988; see Appendix I): NF and Umeå strains of *S. feltiae* at 10, 15, 20 and 25 °C; *S. carpocapsae* All strain at 15, 20 and 25 °C and *S. riobravis* TX strain at 20 and 25 °C.

3.3.3. Upper and Lower Lethal Temperature Tolerance

3.3.3.1. Upper temperature tolerance (UT_{50})

Infective juvenile suspensions of each of the four isolates maintained at the specified temperatures were prepared, with some modifications, as described by Gordon *et al.* (1996). The first infective juveniles (0-48 h- old) to emerge from the insect cadaver were collected into dilute formaldehyde solution inside White traps (Woodring and Kaya, 1988) rinsed twice with distilled water, then suspended in 500 ml distilled water to a concentration of 100-400 infective juveniles/ml. Then, 1 ml of this suspension was transferred into seven separate 50 ml capacity beakers containing 9 ml distilled water. The beakers were then transferred into an incubator (Conviron Controlled Environments Ltd., Winnipeg, Manitoba, Canada) at 26 °C, programmed to increase its temperature automatically by 1 °C every hour. Every hour, a 1 ml infective juvenile suspension was removed from each of the seven beakers, examined under a stereomicroscope and the ratio of dead/live nematodes determined. After counting, each 1 ml sample was poured back into the beaker from which it had been derived. Each beaker was considered as a separate replicate (n= 7).

A basal temperature of 26 °C was selected because it had been observed that, regardless of prior maintenance temperature, all infective juveniles that were transferred to 26 °C were still active after 24 hours. Nevertheless, controls were provided for each experiment. As a control, one separate 50 ml beaker containing 10 ml of the final

infective juvenile suspension (100-400 infective juveniles) of each isolate from each recycling temperature was transferred into an incubator maintained at 26 °C until the end of each experiment and then examined for mortality as described above.

3.3.3.2. Lower temperature tolerance (Lt_{50})

Since it had been determined previously that the infective juveniles of all isolates, regardless of maintenance temperature, were tolerant of cold temperatures down to at least 5 °C, the lower temperature tolerance of infective juveniles was compared by determining the time required to kill 50% (Lt_{50}) of the nematodes at a subzero temperature (-5 °C) rather than by attempting to measure lower lethal temperatures.

Using a Gilson$^{(R)}$ pipetman, 200 (0-48 h- old) pre-rinsed infective juveniles of each isolate were transferred from the distilled water suspension into a set of twenty- four 50 ml beakers containing 10 ml of distilled water. In order to avoid thermal shock, all 24 beakers were transferred to an incubator at 5 °C for 12 hours, 0 °C for another 12 hours, then transferred to -5 °C. The ratio of dead/live nematodes was recorded at 1, 2, 4, 6 and 8 hours after transferring them to -5 °C. For each observation, three beakers containing 200 frozen infective juveniles were removed from the incubator, allowed to thaw and recover for 8 hours at room temperature (25 °C), then the ratio of dead/live nematodes were recorded. All the nematodes in each of the beakers were counted. Each beaker was considered as a separate replicate (n= 3).

In both experiments (UT_{50} and Lt_{50}), the ratio of dead/alive nematodes was

determined on the basis of spontaneous movement and movement after probing immobile nematodes with a lachrymal needle under a stereomicroscope (Gordon *et al.*, 1996).

3.3.3.3. Infectivity of *Steinernema* isolates subsequent to freezing (-5 °C)

Infective juveniles (2500) of each of the four isolates that had been recycled at different temperatures were rinsed, transferred into beakers containing 10 ml distilled water, then transferred gradually to -5 °C as described above. After five hours of freezing, the nematodes were removed from the incubator and allowed to thaw and recover for 8 hours. Two hundred active infective juveniles of each of the four isolates that recovered from freezing were transferred to a filter paper (Whatman no. 4) circle lining the base of each of five separate petri dishes (5 cm dia.) and the filter paper evenly moistened by adding ≤ 0.2 ml distilled water (Gordon *et al.*, 1996). Ten last-instar larvae of *G. mellonella* were added to each petri dish, all of which were then transferred to an incubator maintained at 25 °C. Each petri dish was considered as a separate replicate (n= 5 for each isolate and maintenance temperature).

Larval mortality was assessed 72 hours after infection; all dead insects were transferred to White traps (Woodring and Kaya, 1988) for the emergence of infective juveniles to verify that these insects were infected by nematodes. For each isolate and maintenance temperature, controls consisted of 200 infective juveniles in a single 50 ml beaker containing 10 ml distilled water; control infective juveniles were pre-conditioned to 5 °C as experimental infective juveniles, but were then held at 0 °C in an unfrozen

condition for 5 hours. These infective juveniles were used for infection of wax moth larvae: five replicates (10 insects/ petri dish) were set up for each set of controls.

3.3.4. Statistical analysis

Probit analysis (Norušis, 1990) was used to calculate upper lethal temperature (UT_{50}) and lower lethal temperature time (Lt_{50}) on log_{10} transformed data. In both upper lethal temperature and lower lethal temperature time studies, significant differences between pairs of UT_{50} and Lt_{50} values were based on the criterion of non overlap of 95% confidence limits. Following arcsine transformation, the data on infectivity of *Steinernema* isolates subsequent to freezing was analysed by Kruskal-Wallis non-parametric one way ANOVA (Jandel Corporation(R), Sigma Stat, 1992).

3.4. RESULTS

3.4.1. Upper temperature tolerance

There was no mortality of infective juveniles in any of the controls held at 26 °C. Regardless of species or strain of nematode, the UT_{50} increased progressively with increase in maintenance temperature (Table 1). However, UT_{50} values for each recycling temperature regime were also strain related. The Umeå and NF strains of *S. feltiae*, with lowest UT_{50} values of the four isolates recycled at 20 and 25 °C, possessed the least capacity for upper temperature tolerance, while the TX strain of *S. riobravis* was most

tolerant of high temperature (Fig. 1). Upper lethal temperature (UT_{50}) values for the two strains (NF: Umeå) of *S. feltiae* were not significantly different from each other at all recycling temperature regimes. The All strain of *S. carpocapsae* appeared to display a level of high temperature tolerance intermediate between the two *S. feltiae* strains and *S. riobravis* TX strain. At maintenance temperatures of 15, 20 and 25 °C, the All strain of *S. carpocapsae* had UT_{50} values that were significantly higher than the values for *S. feltiae* Umeå strain; at the 25 °C maintenance temperature, *S. carpocapsae* All strain had a UT_{50} value significantly lower than that of *S. riobravis* TX strain (Fig. 1).

3.4.2. Lower temperature tolerance

There was no mortality of infective juveniles in any of the unfrozen controls held at 0 °C. In all isolates, the survival times at -5 °C increased as maintenance temperature decreased (Table 1). Although *S. feltiae* NF and Umeå strains were more cold tolerant than the other isolates and the only nematodes capable of recycling at 10 °C, their capacity to withstand freezing was impaired by continual recycling at warmer temperatures. Thus, at a maintenance temperature of 15 °C, infective juveniles of *S. carpocapsae* All strain survived freezing at -5 °C as well as did *S. feltiae* infective juveniles (Fig. 2). At maintenance temperatures of 20 and 25 °C, *S. riobravis* TX and *S. carpocapsae* All infective juveniles had Lt_{50} values that were significantly greater than those of the *S. feltiae* strains. The freezing survival time for *S. riobravis* TX that had been recycled at 20 and 25 °C was the same as for *S. carpocapsae* All.

3.4.3. Infectivity of steinernematids subsequent to freezing (-5 °C)

In all control experiments involving the use of unfrozen infective juveniles, 100 % of the insects were killed by the nematodes. In all isolates the maintenance temperature did not significantly affect the infectivity of the infective juveniles that survived freezing (Table 2). Regardless of strain or prior maintenance condition, nematodes that survived freezing for five hours at -5 °C retained over 90% of the infectivity compared to controls. It was also observed that the infective juveniles of each isolate emerged normally from the insect cadaver within 12- 15 days after infection.

3.5. DISCUSSION

This study has shown that the upper and lower thermal tolerances of entomopathogenic nematodes may be experimentally influenced by the temperature at which they are maintained. However, such manipulation of temperature tolerance is restricted by genetic differences among the isolates.

Rearing at warmer temperatures increased the upper lethal temperatures and decreased survival times in the frozen condition in all three species of *Steinernema*. Conversely, when nematodes were reared at colder temperatures, their upper lethal temperatures were decreased, while their freezing survival times were lengthened. The nematodes used in this study were recycled at specified temperature regimes for more than two years. Therefore, the observed shifts in upper and lower temperature tolerances may be due to changes in their genetic constitution resulting from artificial selection and/or to

environmentally - induced processes of thermal acclimation.

With respect to the latter possibility, acclimation to ambient temperatures has been demonstrated for several species of free-living nematodes (Mabbett and Wharton, 1986; Pickup, 1990; Wharton and Brown, 1991), as well as for the transmission stages of animal and plant parasitic ones (Ash and Atkinson, 1986; Forge and MacGuidwin, 1992). Survival at sub-zero temperatures was enhanced in *Heterorhabditis* sp. following 2-4 months storage (Griffin, 1993) or in *H. bacteriophora*, *S. anomali* and *S. feltiae* following short-term (12- 14 days) incubation (Brown and Gaugler, 1996) at temperatures lower than those at which recycling had been carried out. In this study, the possibility that temperature-induced acclimation may, partly, be responsible for the increased thermotolerance of strains that had been recycled at warmer temperatures is supported by Selvan *et al.* (1996), who showed that the infectivity and heat tolerance of *H. bacteriophora* to temperature regimes above the recycling temperature was enhanced by pre-conditioning the infective juveniles to 35 °C for a short time (1- 3 hours). On the basis of long term recycling experiments, Shapiro *et al.* (1996) concluded that the heat tolerance trait of a desert-dwelling strain of *H. bacteriophora* was genetically based, but that the property could be influenced by the rearing temperature.

It would appear that adaptation to cold temperatures in certain entomopathogenic nematodes involves an increased tolerance to freezing of the body fluids (Brown and Gaugler, 1996), while in others, freezing is avoided by depression of the supercooling point (Wharton and Surrey, 1994). However, physiological manifestations of adaptation

to temperatures above freezing include changes in the isozyme profiles of certain metabolic enzymes (Chapter 5) and near the upper lethal temperature, the synthesis of heat shock proteins (Selvan *et al.*. 1996). Further studies are in progress to more fully characterize the physiological nature of the adaptive mechanism(s).

Regardless of the isolate and its maintenance temperature, the infectivity of infective juveniles that survived the freezing regimen was not unduly compromised (< 10%) by the procedure. This suggests that measurement of infective juveniles mortality constitutes a reasonable estimate of the effect of freezing on the nematodes and that alterations in infectivity are not part of the response to sub-zero temperatures. By contrast, prolonged storage (2-4 months) of infective juveniles of *Heterorhabditis* sp. at a cold (9 °C) temperature above zero resulted in improved infectivity at the same temperature, possibly due to cold temperature induced physiological acclimation (Griffin, 1996).

In the present laboratory assays, 50% of the nematodes died within 7 hours subsequent to freezing at -5 °C. This survival percentage appears consistent with those reported for *H. bacteriophora*, *S. anomali*, and *S. feltiae*, viz. 30-40% and 10% after freezing for 12 hours and 1.3-2.7 days, respectively (Brown and Gaugler, 1996). However, such experiments, carried out under varied laboratory conditions, have not been designed to mimic the seasonal changes in soil temperatures that occur in cold climates. As in boreal insects (Sømme, 1982), a gradual sequence of environmental changes may be needed to induce cryoprotectant mechanisms for overwintering. Consequently, while such assays are useful for evaluating comparative tolerances to freezing, the actual values

yielded cannot be directly extrapolated to the field situation.

The current results do not agree with those of Dunphy and Webster (1986), who observed no temperature adaptation. In *H. heliothidis* and two strains of *S. feltiae*, times and infective juveniles concentrations required to kill *G. mellonella* larvae were unaffected by subculturing at various temperatures. These authors employed a shorter (9 months) period of recycling, so it is possible that adaptive mechanisms may not have had sufficient time to develop. Thermal adaptation was reported by Grewal *et al.* (1996) and Shapiro *et al.* (1996) who, as in the present experiment, used a more prolonged recycling period (12 repeated passages through wax moth larvae). Both upper and lower limits for infection of *H. bacteriophora* and *S. anomali* were extended when recycled at the lower (15 °C) and upper (30 °C) thermal limits of their reproduction (Grewal *et al.*, 1996). The adaptive responses that we have described, involving a shift, rather than an expansion, of the tolerance ranges accords more closely with what has been reported to occur in a wide range of other poikilotherms (Fry, 1967; Quinn *et al.*, 1994; Howling *et al.*, 1994).

The fact that genetic differences among the isolates limited the degree to which thermal adaptation occurred is to be anticipated. Species and even strains of entomopathogenic nematodes were found to have distinct temperature limits for development and reproduction (Grewal *et al.*, 1994, 1996; Mason and Hominick, 1995). It has been suggested that the lower and upper temperature limits for the activity of entomopathogenic nematodes are correlated with the temperatures of their original habitats (Molyneux, 1986) and the present studies appear to validate this proposition. With respect

to the isolates used in the present study, the two strains of *S. feltiae*, Umeå and NF, originally isolated from Sweden (Pye and Pye. 1985) and Newfoundland, Canada (Jagdale *et al.*. 1996) respectively, were the only ones able to recycle at 10 °C. *Steinernema riobravis* TX strain could not be recycled at any of the temperatures tested below 20 °C, undoubtedly a reflection of its subtropical habitat (Cabanillas *et al.*, 1994). The All strain of *S. carpocapsae*, an isolate that has been subjected to extensive laboratory and commercial subculturing displayed an intermediate minimum temperature for recycling (15 °C). The degree to which this nematode resembles natural field populations in Georgia, U.S.A., from which it was originally derived (Poinar, 1979), is unknown.

All of the isolates, regardless of recycling temperature, had UT_{50} values (35- 45 °C) that were sufficiently high to satisfy soil temperatures likely to be encountered in all except the most extreme soil situations. Provided that steps are taken to shield infective juveniles from extreme heat and desiccation during their application to the soil, tolerance to high temperatures should not limit their usefulness in pest management. With respect to the use of nematodes in cold climates, however, the present data suggest that the boreally adapted strains of *S. feltiae* would need to be recycled at 10-15 °C in order to retain a high degree of freezing tolerance. The subtropical *S. riobravis* and the commercially produced *S. carpocapsae* All strain recycle well and have a good capacity for tolerating high temperatures, when recycled at temperatures (20- 25 °C) at which commercialization seems practical. Such nematodes appear suited for both inundative and inoculative releases in subtropical/tropical climates in which tolerance to freezing would

not be an issue. Inundative short term applications of these nematodes in temperate or even boreal situations accords with their present usage and with the fact that ability to withstand freezing was compromised in all of the isolates when recycled at 25 °C.

Table 1. Probit analysis of temperature tolerances of three species of *Steinernema* recycled at various temperature regimes.

Species and strains of *Steinernema*	RT[1] (°C)	Slope ± SE	UT^{1}_{50} (°C)	Slope ± SE	Lt^{1}_{50} (Hours)
S. feltiae NF strain	10	38.3 ± 0.7	36.4[a] (36.0 - 36.7)[2]	4.7 ± 0.2	7.3[b] (5.1- 12.9)
	15	56. 8 ± 1.2	38.0[b] (37.5 - 38.4)	6.1± 0.3	6.7[b] (6.1- 10.1)
	20	49.1 ± 1.0	40.2[c] (39.8 - 40.7)	3.3 ± 0.1	3.9[ab] (1.4 - 9.7)
	25	51.2 ± 0.8	40.7[c] (40.3 - 41.1)	4.1 ± 0.1	3.2[a] (2.1 - 4.4)
S. feltiae Umeå strain	10	45.9 ± 1.8	35.0[d] (34.8 - 35.2)	3.5 ± 0.1	6.6[c] (4.9 - 8.1)
	15	36.4 ± 1.1	36.2[e] (35.9 - 36.5)	3.4 ± 0.1	6.3[c] (4.9 - 9.0)
	20	48.2 ± 1.9	37.7[f] (37.4 - 38.0)	3.9 ± 0.1	3.0[d] (3.1 - 4.5)
	25	35.5 ± 1.31	40.4[g] (40.2 - 40.6)	8.7 ± 0.3	2.8[d] (2.5 - 3.0)
S. carpocapsae All strain	15	72.3 ± 1.8	40.2[h] (39.9 - 40.5)	3.8 ± 0.2	6.3[e] (4.8- 10.5)
	20	50.8 ± 0.7	42.4[i] (41.8 - 43.2)	3.5 ± 0.2	6.3[e] (4.8- 10.4)
	25	44.1 ± 1.4	43.4[j] (43.2 - 43.7)	6.4 ± 0.2	3.8[f] (3.1 - 4.1)
S. riobravis TX strain	20	93.4 ± 1.7	44.4[k] (44.5 - 45.2)	3.7 ± 0.2	6.2[g] (4.9- 11.2)
	25	61.6 ± 1.1	46.2[l] (45.9 - 46.5)	5.7 ± 0.2	4.5[h] (3.9-5.0)

[1]RT= Recycling temperature; UT_{50}= Upper lethal temperature; Lt_{50} (hrs) = Lower lethal temperature time.[2]Values in parentheses are the upper and lower 95% confidence intervals. Based on confidence intervals, the values for a given isolate in the same vertical column that are followed by the same letter are not significantly different from one another.

Fig. 1. Effect of recycling temperature on the upper lethal temperature (UT_{50}) of four isolates (= three species) of *Steinernema* maintained at four different temperature regimes. NF = Newfoundland strain of *S. feltiae*; Umeå = Umeå strain of *S. feltiae*; All = All strain of *S. carpocapsae* and TX = TX strain of *S. riobravis*. Vertical lines are standard error bars.

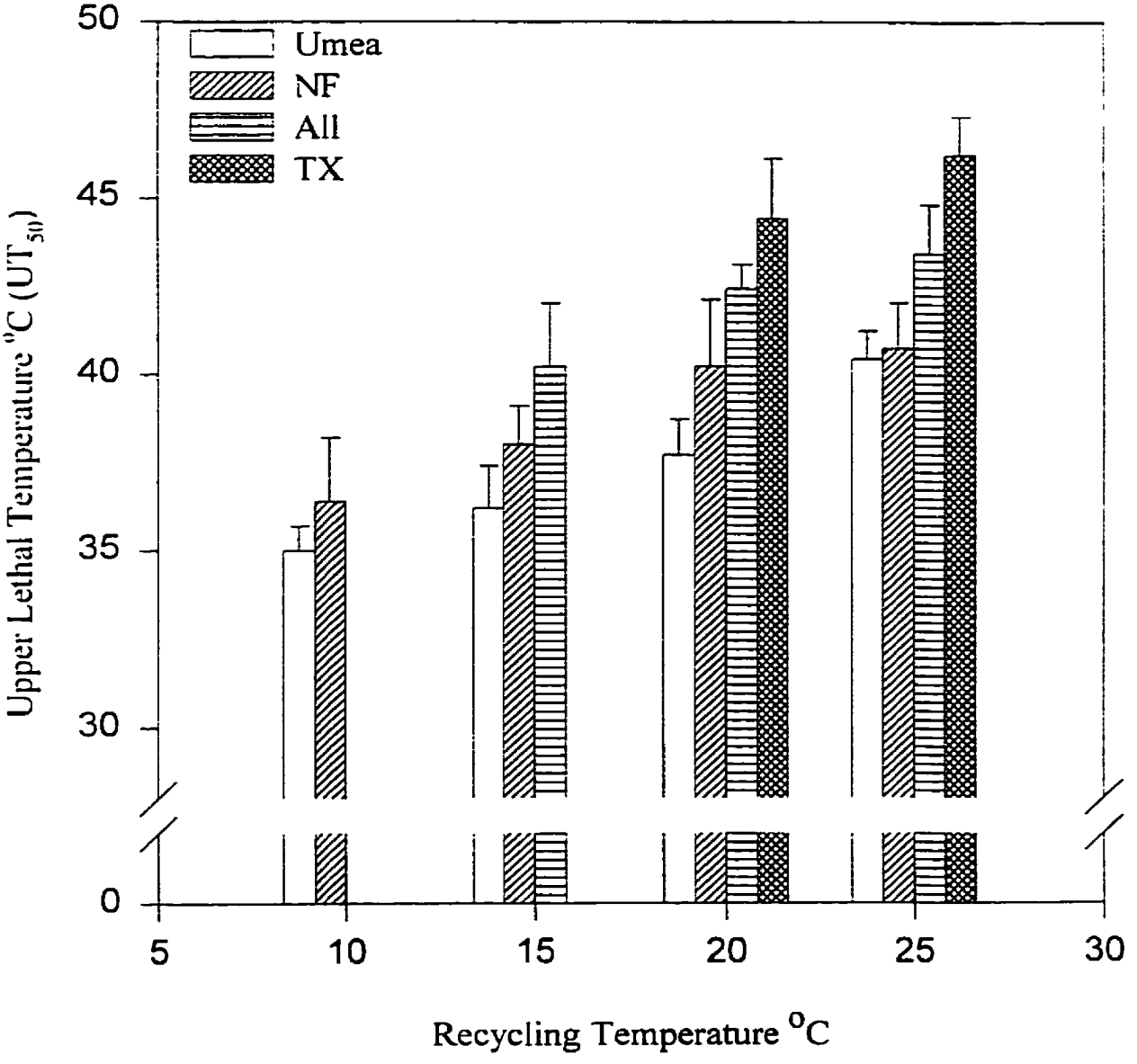

Umea
NF
All
TX
Upper Lethal Temperature °C (UT50)
50
45
40
35
30
0
5
10
15
20
25
30
Recycling Temperature °C

Fig. 2. Effect of four different recycling temperature regimes on the lower lethal temperature time [Lt_{50} (hours)] required to kill 50% steinernematids (three species = four isolates) following 5-hour exposure to -5°C. NF = Newfoundland strain of *S. feltiae*; Umeå = Umeå strain of *S. feltiae*; All = All strain of *S. carpocapsae* and TX = TX strain of *S. riohravis*. Vertical lines are standard error bars.

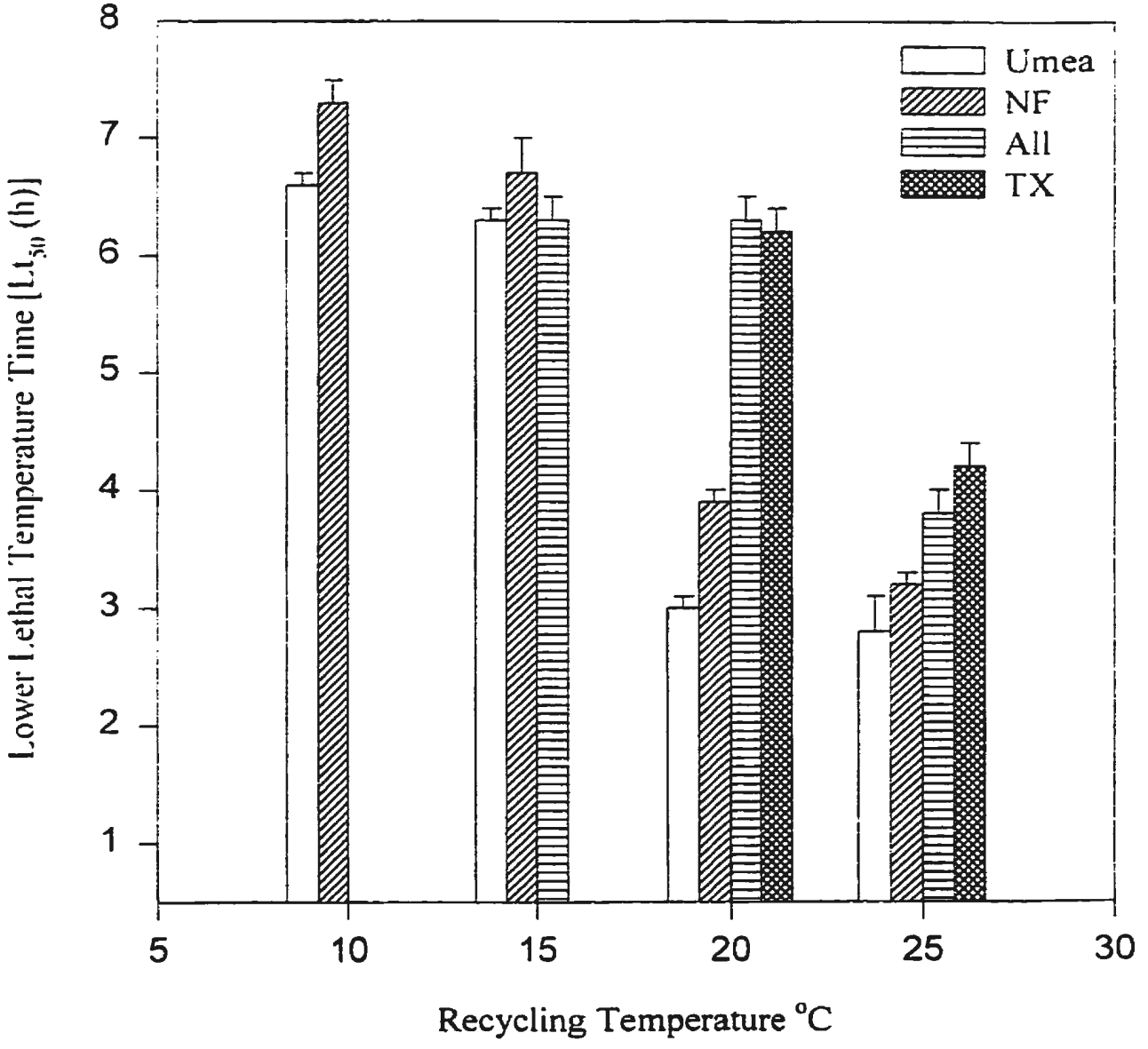

Lower Lethal Temperature Time [LT_{50} (h)]
8
7
6
5
4
3
2
1
5
10
15
20
25
30
Recycling Temperature °C
Umea
NF
All
TX

Table 2. Effect of freezing on the infectivity of entomopathogenic nematodes recycled at various temperatures.

RT (°C)	% Mortality			
	S. feltiae NF strain ± SE	*S. feltiae* Umea strain ± SE	*S. carpocapsae* All strain ± SE	*S. riobravis* TX strain ± SE
10	82.7 ± 4.5[a]	82.7 ± 4.7[b]	-	-
15	81.0 ± 5.6[a]	81.0 ± 5.6[b]	82.6 ± 4.5[c]	-
20	75.4 ± 3.6[a]	81.0 ± 5.6[b]	71.6 ± 0.0[c]	75.6 ± 6.0[d]
25	77.4 ± 5.4[a]	77.4 ± 5.4[b]	71.6 ± 0.0[c]	75.6 ± 6.0[d]

RT= Recycling Temperature. Dashes indicate absence of observations because *S. carpocapsae* and *S. riobravis* did not recycle at these temperatures. Values (Arcsine transformed) followed by the same letter are not significantly different ($P \geq 0.05$), as determined by the Kruskal-Wallis one way ANOVA (n=5).

CHAPTER 4

EFFECT OF RECYCLING TEMPERATURE ON THE INFECTIVITY OF ENTOMOPATHOGENIC NEMATODES.

4.1. ABSTRACT

The effect of long- term (two years) recycling temperatures (10 - 25 °C) on the infectivity of four isolates (*Steinernema carpocapsae* All strain. *Steinernema feltiae* Umeå strain. *Steinernema feltiae* NF strain and *Steinernema riobravis* TX strain) of entomopathogenic nematodes was examined by measuring LD_{50} values in wax moth (*Galleria mellonella*) larvae at bioassay temperatures from 5 to 25 °C. Only the Umeå and NF strains of *S. feltiae* that had been recycled at 10 °C infected and killed the insects at a 5 °C bioassay temperature. *Steinernema carpocapsae* All strain and *S. riobravis* TX strain were infective at 10 °C only when the recycling temperatures were ≤ 20 °C. The infectivity of the two strains of *S. feltiae* at 10 or 15 °C was compromised by propagating them at warmer temperatures (20- 25 °C). The Umeå strain of *S. feltiae* displayed impaired capacity to infect hosts at warm temperatures (20- 25 °C) when recycled at colder (≤ 15 °C) temperatures.

4.2. INTRODUCTION

Entomopathogenic nematodes (f. Steinernematidae and Heterorhabditidae) have potential in controlling a wide range of agriculturally important pests endemic to soil and cryptic environments (Begley, 1990; Klein, 1990). Soil-inhabiting infective juveniles of both steinernematid and hererorhabditid nematodes carry a species- specific symbiotic bacterium of the genera *Xenorhabdus* and *Photorhabdus*, respectively, in their intestines (Boemare *et al.*, 1993). The free-living infective juveniles enter their hosts through natural

openings. Once in the hemocoel. each infective juvenile releases the mutualistic bacteria from its anus. The bacteria release toxins to kill the insect host. usually within 24- 48 hours. then reproduce within the cadaver. providing a nutritional milieu for nematode development and reproduction (Poinar. 1990).

In the commercialization of these nematodes. it is important to determine the degree to which the conditions pertaining to the mass cultivation system affect biological characteristics of the nematode (e.g. temperature tolerance, infectivity) that influence field efficacy. It has been reported that infective juveniles of *Heterorhabditis zealandica* show a greater degree of cold tolerance when cultivated in vivo than when reared in artificial media (Surrey. 1996). It was demonstrated that the capacities of entomopathogenic nematodes to tolerate extreme temperatures may be modified by the temperatures at which they are recycled. Nematodes recycled at colder temperatures have a superior capacity to withstand freezing, but inferior capacity to tolerate warm temperatures. compared with those recycled at warmer temperatures (Chapter 3). Shapiro *et al.* (1996) also showed that the heat tolerance of *Heterorhabditis bacteriophora* IS5 strain was enhanced by recycling the nematodes at 30 °C (compared to 25 °C), whereas no such effects were noted for the commercially available HP88 strain of the same species. Shorter- term temperature-acclimation studies demonstrated that survival at sub-zero temperatures was enhanced in several species of entomopathogenic nematodes following storage at temperatures lower than those at which recycling had been carried out (Brown and Gaugler, 1996; Griffin, 1993). Similarly, the heat tolerance of *H. bacteriophora* to temperature regimes above the

recycling temperature was enhanced by pre-conditioning the infective juveniles to 35 °C for 1-3 hours (Selvan *et al.*, 1996).

To achieve successful inundative and inoculative pest control, it is important that in addition to temperature tolerance, the infectivity of the nematode not be compromised by the rearing protocol. It has been observed that the temperature limits for infectivity and development of *H. bacteriophora* and *Steinernema anomali*, as well as for reproduction of the former species, were extended following prolonged subculturing at temperatures different from the stock cultures (Grewal *et al.*, 1996). Similarly the infectivity and reproduction of *H. bacteriophora* IS5 strain isolated from the Negev desert in Israel were enhanced by recycling the nematodes at 30 °C rather than 25 °C (Shapiro *et al.*, 1996). However, the capacity for infectivity was unaffected in *H. bacteriophora* HP88 strain, *Heterorhabditis heliothidis* and two strains of *S. feltiae* by propagating at various temperatures (Dunphy and Webster, 1986; Shapiro *et al.*, 1996). The present investigation studies the effect of various long- term (two years) recycling temperatures on the infectivity of four isolates of entomopathogenic nematodes, bioassayed at numerous infection temperatures.

4.3. MATERIALS AND METHODS

4.3.1. Sources of Nematodes

Steinernema carpocapsae All strain was provided by Plant Products Ltd.,

Brampton, Ontario, Canada: *S. riobravis* TX strain by Dr. H. E. Cabanillas, USDA, ARS, Crop Insects Research Unit, Weslaco, TX., U. S. A. *Steinernema feltiae* Umeå strain was provided by Dr. R. West, Canadian Forest Service (CFS), St. John's, NF, Canada from a stock colony that had been initially obtained from Biologic Biocontrol Products, Willow Hill, PA, U. S. A. *Steinernema feltiae* NF strain is a new strain that we isolated in Summer 1993 from soil on an organic garden close to St. John's, NF (Jagdale *et al.*, 1996) using *Galleria* bait traps (Woodring and Kaya, 1988). All nematode isolates were recycled once by propagation through wax moth larvae (Woodring and Kaya, 1988) at 25 °C before subjecting them to various recycling regimes.

4.3.2. Effects of recycling temperatures on infectivity

All nematode strains were recycled for two years (May, 1994-May, 1996) by propagation through *G. mellonella* larvae (Woodring and Kaya, 1988; see Appendix I): NF and Umeå strains of *S. feltiae* at 10, 15, 20 and 25 °C; *S. carpocapsae* All strain at 15, 20 and 25 °C and *S. riobravis* TX strain at 20 and 25 °C. Infectivity of these strains from each recycling temperature regime was bioassayed against last-instar larvae of *G. mellonella* at 5, 10, 15, 20 and 25 °C bioassay temperatures and LD_{50} values (i. e. dose of infective juveniles required to kill 50% of the wax moth larvae) determined (Dunphy and Webster, 1986). The sizes of the LD_{50} values are inversely correlated to the nematode's infectivity.

Infective juveniles of each of the five isolates (0-48 h- old) that had been recycled

at different temperatures were transferred from 0.1% formalin solution (40% formaldehyde : distilled water, 1 : 1000 v/v) in the White traps (Woodring and Kaya, 1988) in separate 250-ml beakers containing distilled water and allowed to settle in the beaker. The nematodes were rinsed twice by pouring off the water, adding more distilled water, then pouring off the water again. The nematode suspension was then transferred to a petri dish (9 cm dia.). Using a Gilson$^{(R)}$ pipetman, pre-rinsed infective juveniles of each isolate were individually counted under a stereomicroscope and doses of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 infective juveniles transferred to filter- paper circles (Whatman no. 4) lining the bases of each of five petri dishes (5 cm dia). The filter paper circles were evenly moistened by ≤ 0.2 ml distilled water (Gordon *et al.*, 1996), then all petri dishes containing infective juveniles were transferred to the pertinent infection temperature (5, 10, 15, 20 and 25 °C) for 1 hour. Following such conditioning, ten last-instar larvae of *G. mellonella* were added to each petri dish (Dunphy and Webster, 1986) and maintained at the infection temperature until mortality was assessed. Each petri dish was considered as a separate replicate (n=5). Five replicates were set up for each set of controls at each infection temperature and each control petri dish contained 10 wax moth larvae with no nematodes. Cadavers were dissected to determine the presence of nematodes 3 days, 4 days, 6 days, 10 days and 3 weeks after infection.

4.3.3. Statistical analysis

LD_{50} values [$\log_{10}$ (X + 1) transformed data] were calculated using Probit analysis

(Norušis. 1990). Significant differences between pairs of LD_{50} values were based on the criterion of non-overlap of 95% fiducial limits. There was no mortality in control experiments. so all mortality of infected insects was due to the nematodes and it was unnecessary to apply a correction factor.

4.4. RESULTS

The temperature regimes at which the four nematode isolates were propagated influenced their capacity to infect *G mellonella* larvae over the temperature range (5 - 25 °C) in which infectivity bioassays were conducted (Tables 1-2). Only the two boreal strains of *S. feltiae* were capable of infecting and killing the insects at an infection temperature as low as 5 °C, but such a capacity was very low (LD_{50}, 5-7 times higher than at other temperatures) and only present in nematodes that had been recycled at the coldest (10 °C) temperature (Figs. 1A,B). Similarly, the warm- temperate *S. carpocapsae* All strain and the subtropical *S. riobravis* TX strain were infective at 10 °C only when the recycling temperatures were ≤ 20 °C (Figs. 2A,B).

In situations where infections were possible, the infectivity at lower temperatures of all four isolates was significantly impaired by recycling at warmer temperatures. In *S. feltiae* NF and Umeå strains recycled at 10 and 15 °C, infectivity was the same at all bioassay temperatures from 10 - 25 °C. At higher recycling temperatures (20, 25 °C), infective juveniles of both these isolates had an inferior capacity to infect hosts at 10 °C when compared to that displayed by their cohorts that were bioassayed at higher (20, 25

°C) temperatures (Figs. 1A.B). The infectivity at 10 °C of infective juveniles of *S. feltiae* NF strain that had been propagated at 20 or 25 °C was significantly less (i.e. LD_{50} values significantly higher) than that shown by this strain recycled at 15 °C at any of the bioassay temperatures (Fig. 1A: Table 1). Recycling of *S. carpocapsae* All strain could not be accomplished at 10 °C and recycling of *S. riobravis* TX strain was limited to 20 and 25 °C (Fig. 2 A.B). The infectivity of infective juveniles of *S. carpocapsae* All strain, measured at 10 °C, was the same in nematodes that had been recycled at 20 °C as in those recycled at 15 °C (Fig. 2A). However, nematodes recycled at 25 °C were not infective at 10 °C.

Infectivity of both *S. carpocapsae* All (Fig. 2A) and *S. riobravis* TX (Fig. 2B) strains, measured at ≥ 15 °C, appeared largely unaffected by recycling temperatures when comparisons are made at the same bioassay temperatures between LD_{50} values of infective juveniles recycled at the various temperatures (Table 2). With respect to the two boreal strains of *S. feltiae*, infectivity at 15 °C of the NF strain was significantly impaired by recycling at 25 °C; LD_{50} values (Table 1) of such infective juveniles were significantly higher than those obtained for those recycled at colder temperatures and bioassayed at 15 °C (Fig. 1A). Infectivity of this isolate at 20 and 25 °C was independent of recycling temperature. The capacity of *S. feltiae* Umeå to infect at 15 °C was unaffected by recycling temperature (Fig. 1B). However, infectivity at 20 and 25 °C was significantly compromised by recycling at temperatures ≤ 15 °C; LD_{50} values for nematodes recycled at 10 and 15 °C were significantly higher than those of their counterparts that had been

recycled at warmer temperatures and bioassayed at 20 and 25 °C (Table 1).

4.5. DISCUSSION

This study has shown that the temperatures at which four strains of *Steinernema* were recycled influenced the capacity of the nematodes to infect wax moth larvae at bioassay temperatures from 5 to 25 °C. Infection at the lower limit of each isolate's temperature range for infection was prevented by recycling at warmer temperatures. Thus, the NF and Umeå strains of *S. feltiae* that were recycled at ≥ 15 °C were unable to infect hosts at 5 °C, although these strains were capable of infecting and killing hosts at such a temperature when propagated at 10 °C. Similarly, infective juveniles of *S. carpocapsae* All strain and *S. riobravis* TX strain that had been recycled at 25 °C were unable to infect wax moth larvae at 10 °C, although infection at this temperature occurred when nematodes had been recycled at lower temperatures (All strain: 15 and 20 °C; TX strain: 20 °C).

In addition to completely suppressing infection, partial impairment of infectivity at low bioassay temperatures occurred in the two strains of *S. feltiae* that had been propagated at warmer temperatures. Diminished infectivity at 10 °C characterized both isolates following propagation at 20 and 25 °C; the infectivity of the NF strain at 15 °C was compromised by propagation at 20 °C. The converse effect of recycling temperatures, i.e. impaired capacity to infect at warm temperatures (20- 25 °C) caused by recycling at colder (≤ 15 °C) temperatures, was displayed by *S. feltiae* Umeå strain.

The dependence of infectivity upon recycling temperatures, as displayed by the

four isolates in this study, may be of more general occurrence among entomopathogenic nematodes. Grewal *et al.* (1996) showed that the temperature range over which infections could occur in *H. bacteriophora* and *S. anomali* were extended, in comparison with the normal recycling temperature (25 °C), by propagating nematodes at either 15 or 30 °C. Similarly, *H. bacteriophora* IS5 strain that had been recycled (12 passages) at 30 °C had greater heat tolerance and increased capacity to infect wax moth larvae at 30 °C compared with nematodes that had been recycled at 25 °C (Shapiro *et al.*, 1996). By contrast, however, Dunphy and Webster (1986) found that the infectivity of *H. heliothidis* NC-1 strain and *S. feltiae* DD136 and Mexican strains was not affected by subculturing for a shorter period (9 months) at various temperatures.

The changes in infectivity observed in this study may be genetically based, i.e., the result of long- term recycling involving artificial selection. Shapiro *et al.* (1996) concluded that the heat- tolerance trait of a desert- dwelling strain (IS5) of *H. bacteriophora* was genetically based, but that the property could be influenced by the environmental temperature. The possibility that the changes in infectivity could be partly, environmentally induced is supported by the studies of Griffin (1996), who showed that storage (2-4 months) of infective juveniles of *Heterorhabditis* sp. at a cold (9 °C) temperature resulted in improved infectivity at the same temperature.

Regardless of the nature of the induction mechanism, genetic and/or otherwise, the changes in infectivity observed in this study are part of a broader range of mechanisms deployed by the nematodes for compensating to the recycling temperature regimes.

Nematodes recycled at colder temperatures were found to have superior capacities to withstand freezing, but inferior capacities to tolerate warm temperatures, compared with those recycled at warmer temperatures (see Chapter 3).

The findings of this study have practical significance in the implementation of insect management programs involving steinernematids that result in good infectivity in the field. If *S. carpocapsae* All strain or *S. riobravis* TX strain are to be used in situations where the soil temperature is as low as 10 °C (e.g. in a boreal zone during the early Summer period), infective juveniles should be administered that have been recycled at ≤ 20 °C, recognizing that it would not be possible to recycle the TX isolate at 15 °C. Use of these isolates at soil temperatures ≥ 15 °C would not be affected by the recycling temperature that we have evaluated. In the unlikely possibility that field infections at 5 °C are desired, only the two boreal *S. feltiae* strains could be used, infectivity would be low and nematodes that had been recycled at 10 °C would be required. Field infections at 10 - 15 °C would not be affected by the recycling temperature regime in the case of *S. feltiae* Umeå strain. However, use of the NF strain of *S. feltiae* at 10 °C would be best accomplished using infective juveniles that had been recycled at ≤ 15 °C, while 15 °C field infections should utilize nematodes recycled at ≤ 20 °C. Where soil temperatures are in the 20 - 25 °C range, the NF strain of *S. feltiae* could be used without reference to the recycling temperatures that were screened in this study. However, the Umeå strain of *S. feltiae* should be administered from stocks that had been recycled at ≥ 20 °C, which would have the best infectivity at these field temperatures.

Although the infectivity and presumably, field efficacy, of steinernematids can be modified by the recycling temperature, geographic and consequent genetic isolation of the various species and strains would be expected to play a primary role in determining the choice of nematode to be used in pest management. It has been suggested that the temperature limits for the activity of different species of entomopathogenic nematodes are correlated with the environment of their original habitat (Molyneux, 1986). With respect to the isolates used in the present study, the two boreal strains of *S. feltiae*, NF and Umeå, originally isolated from northern Sweden (Pye and Pye, 1985) and Newfoundland, Canada (Jagdale *et al.*, 1996), respectively, were the only ones able to recycle at 10 °C and infect hosts at 5 °C. *Steinernema riobravis* TX strain a subtropical isolate from southern Texas, U.S.A. (Cabanillas *et al.*, 1994), could not be recycled at any of the temperatures tested below 20 °C and the All strain of *S. carpocapsae*, an isolate that was originally recovered from a warm temperate (Georgia, U.S.A.) habitat (Poinar, 1979) displayed an intermediate minimum (15 °C) temperature for recycling. Both of these nematodes displayed poor or no infectivity at a 10 °C bioassay temperature. These observations give support to the proposition by Griffin (1993) that pre-adapted wild-type populations should be selected from habitats that have similar climatic profiles to those in which use is envisaged.

Table 1. Probit analysis of infectivity of two strains of *S. feltiae* from various recycling temperature regimes at different bioassay temperatures

RT[1]	BT[1]	*Steinernema feltiae* NF strain		*Steinernema feltiae* Umeå strain	
°C	°C	Slope ± SE	LD^1_{50}	Slope ± SE	LD^1_{50}
10	5	1.9 ± 0.3	17.3 (12.8 - 31)^{a}	1.1 ± 0.2	26.8 (15.5 - 101)^{a}
	10	1.0 ± 0.2	3.3 (2.3 - 4.2)^{bce}	1.8 ± 0.2	2.8 (1.8 - 3.7)^{bcde}
	15	1.4 ± 0.2	2.4 (0.9 - 3.6)^{b}	2.2 ± 0.2	2.5 (1.4 - 3.4)^{bcde}
	20	2.4 ± 0.2	3.8 (3.0 - 4.6)^{bce}	2.7 ± 0.2	3.3 (2.5 - 4.0)^{bc}
	25	1.6 ± 0.2	3.9 (3.3 - 4.6)^{bc}	1.7 ± 0.2	3.6 (3.0 - 4.2)^{bc}
15	5	0.0 ± 0.0	NI[1]	0.0 ± 0.0	NI
	10	1.8 ± 0.2	3.0 (2.1 - 2.9)^{b}	2.1 ± 0.2	2.6 (2.2 - 3.9)^{bcd}
	15	1.5 ± 0.2	2.8 (1.6 - 3.8)^{b}	2.4 ± 0.2	2.5 (2.1 - 2.9)^{b}
	20	2.3 ± 0.2	3.3 (2.8 - 3.7)^{bc}	2.0 ± 0.2	3.0 (2.2 - 3.9)^{bcd}
	25	2.8 ± 0.2	3.4 (2.7 - 4.0)^{bc}	2.6 ± 0.2	3.3 (2.3 - 4.3)^{bcd}
20	5	0.0 ± 0.0	NI	0.0 ± 0.0	NI
	10	2.2 ± 0.2	5.5 (4.1 - 7.6)^{cd}	1.8 ± 0.2	4.1 (3.5 - 4.8)^{c}
	15	1.8 ± 0.2	3.5 (2.9 - 4.0)^{bc}	2.2 ± 0.2	2.9 (1.7 - 3.9)^{bcde}
	20	1.3 ± 0.2	2.7 (2.0 - 3.4)^{bc}	1.8 ± 0.2	1.9 (1.0 - 2.7)^{def}
	25	1.4 ± 0.2	2.3 (0.8 - 3.5)^{bc}	2.1 ± 0.2	1.7 (0.9 - 2.4)^{def}
25	5	0.0 ± 0.0	NI	0.0 ± 0.0	NI
	10	1.0 ± 0.9	7.3 (5.6 - 10)^{d}	1.3 ± 0.2	4.5 (3.6 - 5.5)^{c}
	15	2.6 ± 0.3	5.7 (5.2 - 6.4)^{d}	1.7 ± 0.2	2.8 (1.5 - 3.8)^{bcdef}
	20	1.8 ± 0.2	2.6 (1.8 - 3.4)^{bc}	2.5 ± 0.2	1.4 (0.9 - 1.8)^{ef}
	25	0.7 ± 0.1	2.1 (0.8 - 3.2)^{c}	1.6 ± 0.2	0.9 (0.5 - 1.2)^{f}

[1]RT = Recycling temperature; BT = Bioassay temperature; LD_{50} = Infective juveniles required to kill 50% wax moth larvae; NI = No infection. Values in parenthese are the upper and lower 95% confidence intervals. Based on confidance intervals, the values for a given isolate in the same vertical column that are followed by the same letter are not significantly different from one another.

Fig. 1. Effect of recycling temperatures on the infectivity (LD_{50} values) of NF (A) and Umeå (B) strains of *S. feltiae* at various bioassay temperatures. BT = Bioassay temperature. NI = No infection. Bars with the same letter(s) are not significantly different.

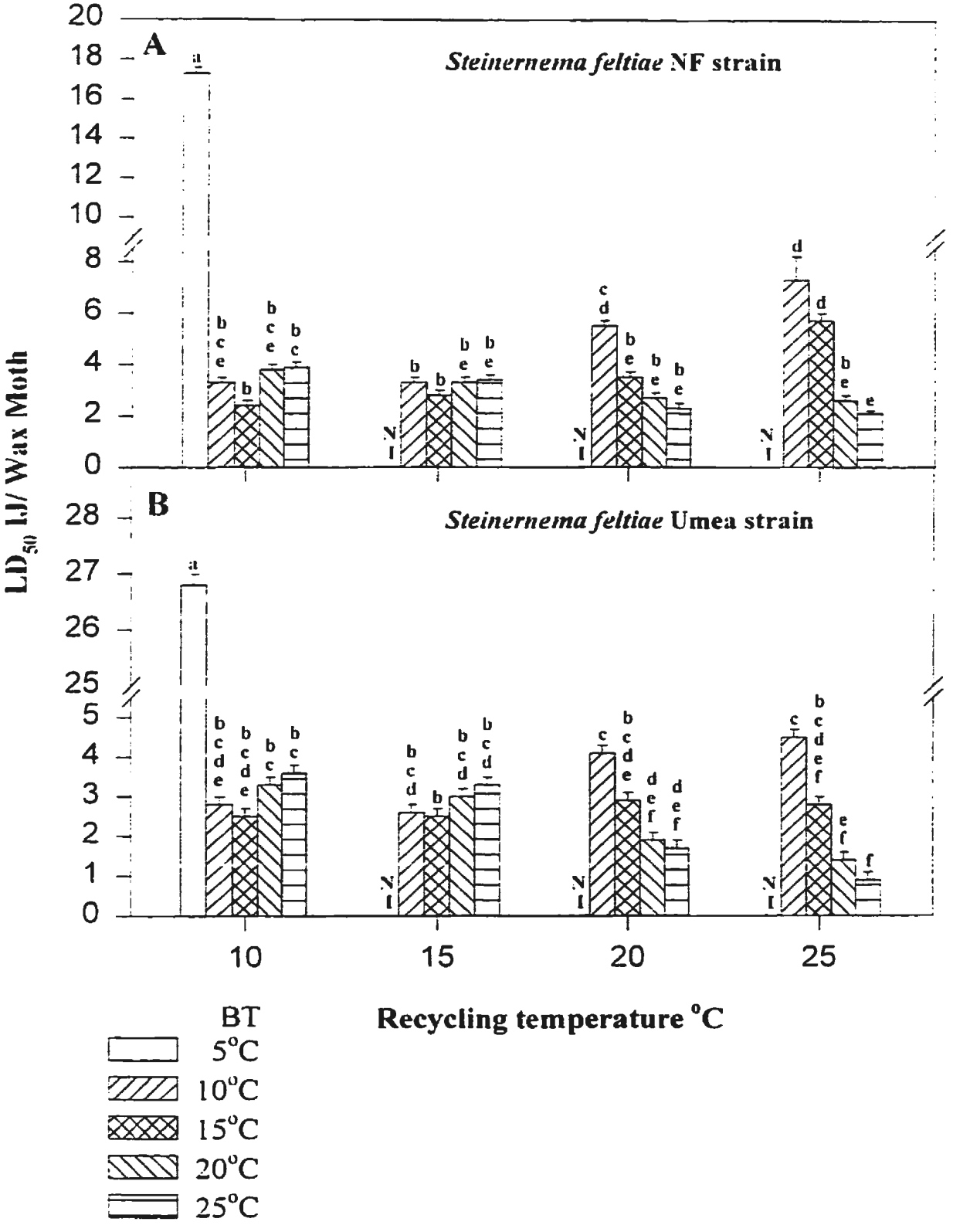
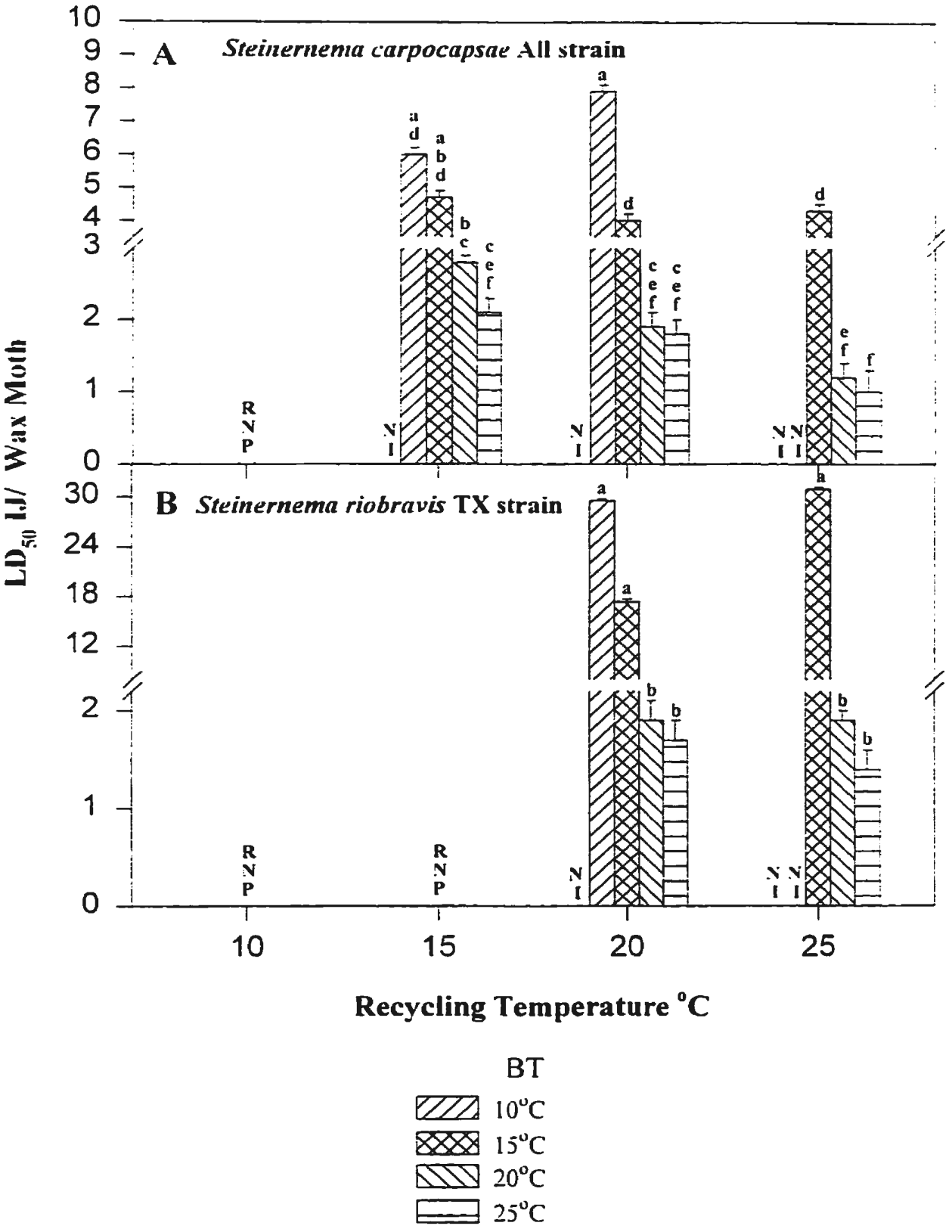

A
Steinernema feltiae NF strain
B
Steinernema feltiae Umea strain
LD50 IJ/ Wax Moth
Recycling temperature °C
BT
5°C
10°C
15°C
20°C
25°C

Table 2. Probit analysis of infectivity of *S. carpocapsae* All strain and *S. riobravis* TX strain from various recycling temperature regimes at different temperature.

RT[1]	BT[1]	*Steinernema carpocapsae* All strain		*Steinernema riobravis* TX strain	
°C	°C	Slope ± SE	LD^1_{50}	Slope ± SE	LD^1_{50}
10	5	-	-	-	-
	10	-	-	-	-
	15	-	-	-	-
	20	-	-	-	-
	25	-	-	-	-
15	5	0.0 ± 0.0	NI[1]	-	-
	10	1.9 ± 0.2	6.0 (4.8 - 7.8)^{ad}	-	-
	15	1.8 ± 0.2	4.7 (3.4 - 6.4)^{abd}	-	-
	20	1.2 ± 0.1	2.8 (2.0 - 3.5)^{bc}	-	-
	25	1.6 ± 0.2	2.1 (1.3 - 2.8)^{cef}	-	-
20	5	0.0 ± 0.0	NI	0.0 ± 0.0	NI
	10	1.7 ± 0.2	7.9 (6.2 - 11)^{a}	0.9 ± 0.2	29.5 (15.8 - 155.0)^{a}
	15	1.4 ± 0.2	4.0 (3.2 - 4.9)^{d}	1.4 ± 0.3	17.3 (12.2 - 33.9)^{a}
	20	1.9 ± 0.2	1.9 (1.1 - 2.7)^{cef}	2.2 ± 0.2	1.9 (1.6 - 2.3)^{b}
	25	2.2 ± 0.2	1.8 (1.5 - 2.2)^{ce}	2.1 ± 0.2	1.7 (1.3 - 2.0)^{b}
25	5	0.0 ± 0.0	NI	0.0 ± 0.0	NI
	10	0.0 ± 0.0	NI	0.0 ± 0.0	NI
	15	1.9 ± 0.2	4.3 (2.8 - 6.1)^{d}	1.4 ± 0.2	30.9 (15.0 - 211.0)^{a}
	20	1.6 ± 0.2	1.2 (0.8 - 1.6)^{ef}	0.8 ± 0.1	1.9 (0.8 - 2.8)^{b}
	25	2.6 ± 0.3	1.0 (0.7 - 1.3)^{f}	2.1 ± 0.2	1.4 (1.0 - 1.8)^{b}

[1]RT = Recycling temperature; BT = Bioassay temperature; LD_{50} = Infective juveniles required to kill 50% wax moth larvae; NI = No infection. Values in parenthese are the upper and lower 95% confidence intervals. Based on confidance intervals, the values for a given isolate in the same vertical column that are followed by the same letter are not significantly different from one another. Dashes indicate that the recycling of these strains was not possible at that perticular temperature.

Fig. 2. Effect of recycling temperatures on the infectivity (LD_{50} values) of *S. carpocapsae* All strain (A) and *S. riobravis* TX strain (B) at various bioassay temperatures. BT = Bioassay temperature. NI = No infection. RNP = Recycling not possible. Bars with the same letter(s) are not significantly different.


A Steinernema carpocapsae All strain
B Steinernema riobravis TX strain
LD50 IJ/ Wax Moth
Recycling Temperature °C
BT
10°C
15°C
20°C
25°C

CHAPTER 5

EFFECT OF TEMPERATURE ON THE SYNTHESIS OF ISOZYMES IN ENTOMOPATHOGENIC NEMATODES.

5.1. ABSTRACT

Cellulose acetate electrophoresis was used to determine the degree to which isozyme banding patterns differed among isolates of steinernematid nematodes that had been recycled or stored at 5, 10, 15, 20 and 25 °C: *Steinernema feltiae* Umeå strain, *S. carpocapsae* All strain, *S. riobravis* TX strain and *S. feltiae* NF strain. In all four isolates, isozyme banding patterns of malate dehydrogenase (MDH), mannose-6-phosphate isomerase (MPI) and phosphoglucomutase (PGM) were affected by recycling or storage temperature. However, the isozyme distribution patterns of glycerol-3-phosphate dehydrogenase (G3PDH) were not temperature related.

The NF and Umeå strains of *S. feltiae* synthesized additional isozymes of MPI and MDH or PGM in response to cold temperatures, while *S. carpocapsae* All strain synthesized three isozymes of MDH in response to warm temperatures and an additional isozyme of PGM in response to cold temperatures. Isozyme synthesis may constitute a temperature adaptation mechanism for these nematodes.

5.2. INTRODUCTION

The reproduction, development, dispersal and infectivity of entomopathogenic nematodes (f. Steinernematidae and Heterorhabditidae) are affected by environmental temperature (Kaya, 1977; Grewal *et al.*, 1994; Mason and Hominick, 1995; Griffin, 1993; Steiner, 1996). Nevertheless, infective juveniles of different species of entomopathogenic nematodes have been recovered from the soils of a wide variety of climatic regions

(Amarasinghe *et al.*, 1994; Cabanillas *et al.*, 1994; Boag *et al.*, 1992; Griffin *et al.*, 1991; Jagdale *et al.*, 1996; Mràček and Webster, 1993).

However, nothing is known about the physiological mechanisms involved in temperature adaptation of the nematodes. The thrust of research on temperature adaptation mechanisms in poikilotherms has been on teleosts and to a lesser degree, echinoderms (Marcus, 1977), which deploy a medley of strategies, including the synthesis of isozymes that are functionally suited for specific temperature regimes (Cossins, 1983; Hazel, 1995; Hazel and Prosser, 1974; Hochachka and Somero, 1984; Johnston, 1983; Lin *et al.*, 1996; Sidell, 1983; Somero, 1995). The degree to which this temperature adaptation strategy is used by other groups of poikilotherms, including nematodes, has not been thoroughly investigated.

The purpose of the present investigation was to determine whether there was a change in the isozyme patterns of four key metabolic enzymes extracted from the four strains of *Steinernema*, which had been maintained or stored in the laboratory for prolonged period of time at various temperature regimes.

5.3. MATERIALS AND METHODS

5.3.1. Sources of nematodes

Steinernema carpocapsae All strain was provided by Plant Products Ltd., Brampton, Ontario, Canada; *S. riobravis* TX strain by Dr. H. E. Cabanillas, USDA, ARS,

Crop Insects Research Unit, Weslaco, TX., U.S.A. *Steinernema feltiae* Umeå strain was provided by Dr. R. West, Canadian Forest Service, St. John's, NF, Canada from a stock colony that had been initially obtained from Biologic Biocontrol Products, Willow Hill, PA., U.S.A. *Steinernema feltiae* NF strain is a new strain (Jagdale *et al.*, 1996) that we isolated in Summer 1994 from soil on an organic garden close to St. John's, NF, using *Galleria* bait traps (Woodring and Kaya, 1988).

5.3.2. Recycling/storage temperature regimes

All nematode strains were recycled at different temperature regimes for two years (May, 1994-May, 1996) by propagation through wax moth (*Galleria mellonella*) larvae (Woodring and Kaya, 1988, see Appendix I): NF and Umeå strains *of S. feltiae* at 10, 15, 20 and 25 °C; All strain of *S. carpocapsae* at 15, 20 and 25 °C and TX strain of *S. riobravis* at 20 and 25 °C. Infective juveniles of these nematodes were stored in tissue culture bottles (600 ml) for four months (February, 1996-May, 1996) at temperatures where recycling of isolates was not possible (*S. feltiae* at 5 °C, *S. carpocapsae* at 5 and 10 °C. *S. riobravis* at 5, 10 and 15 °C).

5.3.3. Cellulose acetate electrophoresis

The procedures for enzyme extraction, cellulose acetate electrophoresis and enzyme staining were as described in Chapter 2 (Jagdale *et al.*, 1996). The enzymes were extracted from the infective juveniles of each of the four isolates (40 mg wet weight)

recycled or stored at the temperature regimes employed for each isolate.

Specific stains were used to visualize the following enzymes: glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), malate dehydrogenase (EC 1.1.1.37), mannose-6-phosphate isomerase (EC 5.3.1.8) and phosphoglucomutase (EC 2.7.5.1) (Hebert and Beaton, 1993).

Three replicate plates were prepared for each nematode isolate, each replicate representing a separate infective juveniles homogenate. The relative electrophoretic mobility (μ) for each isoenzyme was measured to compare the migration rates (Lehninger, 1979) and isozyme bands among temperature regimes were considered the same if their μ values were within 10% of one another. This margin of error was selected because the highest and lowest μ values among three replicates of the same isozyme were always found to be within 10% of one another (Jagdale *et al.*, 1996).

5.4. RESULTS

5.4.1. Glycerol-3-phosphate dehydrogenase (G3PDH)

The isozyme distribution patterns of this enzyme were not temperature related. All isolates that have been maintained or stored at 5, 10, 15, 20 and 25 °C possessed a single band of G3PDH activity that migrated toward the cathode (Fig. 1 A-D; Table 1).

5.4.2. Mannose-6-phosphate isomerase (MPI)

Isozymes of this enzyme extracted from *S. carpocapsae* All strain and *S. riobravis*

TX strain were not affected by temperature. Each of these species of *Steinernema* possessed only two cathodal isozyme bands which were common to all temperature regimes (Table 1). The isozyme profile of this enzyme was temperature related for the NF and Umeå strains of *S. feltiae*. The NF strain of *S. feltiae* possessed six cathodal bands of MPI activity at 5 °C, four bands at 20 °C and only three bands at 10, 15, 20, and 25 °C. These three isozymes were common to all temperature regimes. Thus, three additional isozymes were synthesized at 5 °C, one of which was also synthesized at 20 °C (Fig. 1 E; Table 1). The Umeå strain of *S. feltiae* possessed three common isozyme bands at 5, 10, 15 and 20 °C. At 25 °C, only two of these enzymes were synthesized (Fig. 1 F).

5.4.3. Phosphoglucomutase (PGM)

The isozyme distribution patterns of this enzyme extracted from *S. feltiae* Umeå strain and *S. riobravis* TX strain were not temperature related. Each of these species possessed two cathodal bands regardless of the temperature regime at which they were maintained. (Table 1). However, the isozymes of this enzyme were temperature related for the All strain of *S. carpocapsae* (Band- 2) and the NF strain of *S. feltiae* (Table 1). *Steinernema carpocapsae* All strain recycled at 20 and 25 °C possessed three isozymes (Table 1). At lower temperatures (5, 10, 15 °C), an additional isozyme (the same one at all three temperatures) was synthesized (Fig. 2 A). *Steinernema feltiae* NF strain possessed four isozymes of PGM common to all recycling and storage temperatures. However, one additional isozyme was synthesized by nematodes recycled at 10 °C (Table 1) or stored

at 5 °C (Fig. 2 B). The additional isozyme synthesized at 5 °C had a different electrophoretic mobility from the one synthesized at 10 °C.

5.4.4. Malate dehydrogenase (MDH)

The isozyme distribution patterns of MDH were temperature related for all four isolates. *Steinernema carpocapsae* All strain recycled at 25 °C possessed four isozymes, whereas only one such isozyme was produced at the other temperatures (Fig. 2 C; Table 1). The NF strain of *S. feltiae* possessed two (5; 25 °C), three (10; 15 °C), or four (20 °C) cathodal bands of MDH activity, according to storage or recycling temperature; one of these isozymes was common to all temperature regimes (Fig. 2 D; Table 1). The Umeå strain of *S. feltiae* possessed two common isozymes when recycled at 5, 10 and 15 °C, whereas only one isozyme was present when recycled at higher temperature regimes (20; 25 °C) (Fig. 2 E). *Steinernema riobravis* TX strain recycled at 25 °C had one isozyme, two bands of MDH activity at 5 and 20 °C and three isozymes at 10 and 15 °C (Fig. 2 F; Table 1).

5.5. DISCUSSION

The steinernematid isolates responded differentially to temperature with respect to synthesis of isozymes. Isozyme patterns of MDH, MPI and PGM were temperature related in four (All, NF, Umeå and TX), two (NF and Umeå) and two (All and NF) strains of steinernematid nematodes, respectively. *Steinernema feltiae* NF strain displayed

temperature related changes in isozyme synthesis for three enzymes (MDH, MPI, PGM), *S. feltiae* Umeå strain for two enzymes (MDH, MPI), *S. carpocapsae* All strain for two enzymes (MDH, PGM) and *S. riobravis* TX strain for one enzyme (MDH). None of the isolates showed temperature related modifications in synthesis of isozymes for the enzyme G3PDH.

The production of isozymes, with kinetic properties suited to the ambient temperature, is regarded as an important component of the seasonal temperature adaptation mechanism in teleosts (Baldwin and Hochachka, 1970; Lin *et al.*, 1996; Lin and Somero, 1995; Moon and Hochachka, 1971; Yamawaki and Tsukuda, 1979) and echinoderms (Marcus, 1977). Experimental evidence for such a strategy is based on studies that have demonstrated the production of one or other of two isozymes of a given enzyme, depending upon the temperature at which the animals were maintained thus extending the range of optimal activity of the enzyme. The nematodes examined in the present study yielded isozyme data that were not so clear cut, attributed at least in part to the fact that most of the enzymes were divisible into more than two isozymes. This situation notwithstanding, most of the temperature induced changes in isozyme composition are explicable as adaptive in nature. Thus, it is likely that *S. carpocapsae* All strain synthesized three additional isozymes of MDH at 25 °C as an adaptation to warm temperature and one additional isozyme of PGM at temperatures ≤ 15 °C as an adaptation to colder temperatures. *Steinernema feltiae* NF strain synthesized three additional isozymes of MPI at 5 °C and one additional isozyme of PGM at 10 and 5 °C (not the same isozyme

at these two temperatures) as adaptations to cold temperature. *Steinernema feltiae* Umeå strain synthesized one additional isozyme of MPI at temperatures ≤ 20 °C and one additional isozyme of MDH at temperatures ≤ 15 °C to adapt to colder temperatures.

However, interpretation of other temperature related data is less clear. *Steinernema riobravis* TX strain yielded zymograms for MDH that were identical only at 10 and 15 °C; at temperatures above and below this range, there was a decrease in the number of isozymes. Also with respect to MDH, *S. feltiae* NF strain possessed an isozyme distribution pattern that displayed no observable trend, except that it was the same only for nematodes maintained at 10 and 15 °C. Thus, it is proposed that most of the enzymes investigated in the current study in the nematodes responded to changes in storage or recycling temperatures by shifting the synthesis of isozymes in an adaptive fashion. In situations where isozyme synthesis was temperature related, but where directional trends were not obvious, further studies are needed to more fully understand the functional significance of the observed changes.

From the limited studies conducted, it would appear that the NF and Umeå strains of *S. feltiae*, isolated from Newfoundland (Jagdale *et al.*, 1996) and Sweden (Pye and Pye, 1985) respectively, displayed changes in isozyme synthesis geared toward cold adaptation, as would be anticipated from their boreal habitats. *Steinernema carpocapsae* All strain, a nematode that has been repeatedly subcultured in the laboratory for many years, displayed changes in isozyme profile conducive to both cold and warm temperatures. While the present study revealed no changes in the isozyme profiles of *S. riobravis* TX

that could be viewed as adaptive to its subtropical habitat. only four enzymes were examined and further studies are required to elucidate mechanisms of heat tolerance in this nematode. It is possible that this particular nematode does not have as greater requirement for temperature adaptation mechanism as in the other strains studied. because soil in its sub-tropical habitat constitute more stable temperature.

In studies done on temperature adaptation in teleosts, animals were held for relatively short periods of time (usually several weeks) at stable, predetermined temperature regimes and isozyme studies conducted on the same organisms as were initially set up (Somero, 1995). No reproduction occurred. Accordingly, changes in isozyme profile were attributed to environmentally- induced thermal acclimation. However. in the present study, many of the nematodes were recycled over a prolonged time frame of two years. In such cases, further studies would be required to determine whether changes in isozyme distribution pattern are environmentally- induced or whether they resulted from genetic selection. This would apply, for example, to all changes in isozyme distribution patterns recorded for *S. carpocapsae* All and *S. feltiae* Umeå, which were recycled at each of the temperatures at which changes in isozyme distribution pattern occurred. However, the synthesis of new isozymes of MPI and PGM by *S. feltiae* NF strain as well as loss of MDH isozyme synthetic capacity by *S. riobravis* at 5 °C must have been environmentally- induced, because the nematodes were stored but not recycled at this temperature.

The enzymes examined in this study are involved in key pathways of carbohydrate

catabolism in Metazoa. Glycerol 3-phosphate dehydrogenase and malate dehydrogenase are necessary for the glycerol phosphate shuttle and the malate-aspartate shuttle, respectively and these systems are crucial for aerobic glycolysis. Additionally, MDH is involved in mediating pathways that connect to glycolysis (viz. tricarboxylic cycle and fumarate reductase pathways), while MPI and PGM facilitate glycolysis and glycogenolysis, respectively (Lehninger, 1979). The full significance of modulating such pathways in response to temperature can only be appreciated when more information is obtained on the biochemical ecology of these nematodes.

From a purely practical standpoint, it is proposed that cellulose acetate electrophoresis could be a convenient taxonomic tool for identifying species, possibly strains, of steinernematids (Jagdale *et al.*, 1996). On the basis of the present study, it is important to stress that recycling and storage temperatures should be standardized when carrying out such identifications.

Fig. 1. Electropherograms of two enzymes in three species (four isolates) of *Steinernema* recycled stored at five different temperature regimes. A-D: G3PDH = Glycerol-3-phosphate dehydrogenase; E-F: MPI = Mannose-6-phosphate isomerase. Arrow heads indicate points of sample application. Bands were numbered in increasing numerical order relative to the distance that they migrated cathodally from the origin.

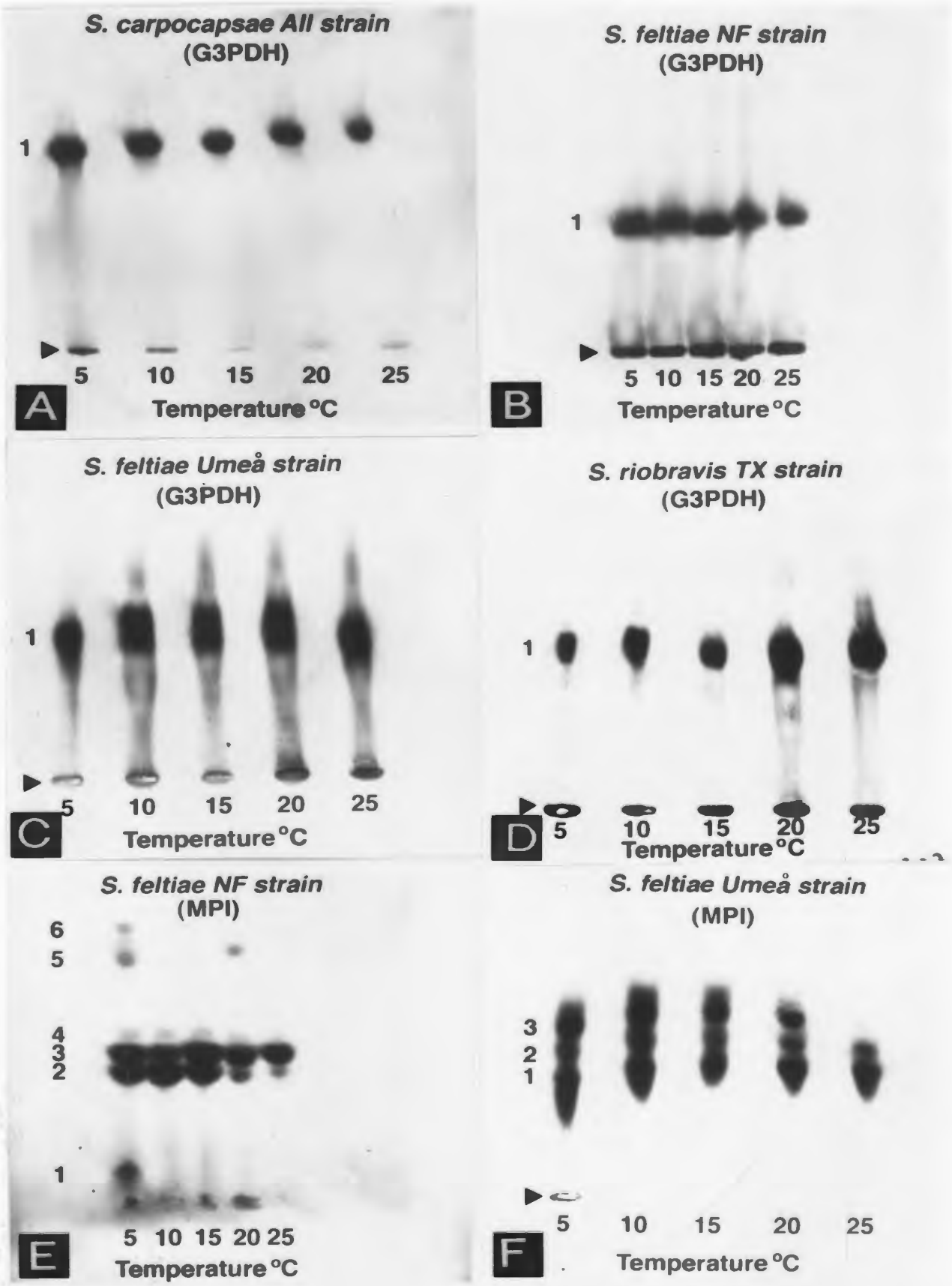

S. carpocapsae All strain
(G3PDH)
1
5 10 15 20 25
A
Temperature °C
S. feltiae NF strain
(G3PDH)
1
5 10 15 20 25
B
Temperature °C
S. feltiae Umeå strain
(G3PDH)
1
5 10 15 20 25
C
Temperature °C
S. riobravis TX strain
(G3PDH)
1
5 10 15 20 25
D
Temperature °C
S. feltiae NF strain
(MPI)
6
5
4
3
2
1
5 10 15 20 25
E
Temperature °C
S. feltiae Umeå strain
(MPI)
3
2
1
5 10 15 20 25
F
Temperature °C

Fig. 2. Electropherograms of two enzymes in three species (four isolates) of *Steinernema* recycled stored at five different temperature regimes. A-B: PGM = Phosphoglucomutase: C-F: MDH = Malate dehydrogenase. Arrow heads indicate points of sample application. Bands were numbered in increasing numerical order relative to the distance that they migrated cathodally from the origin

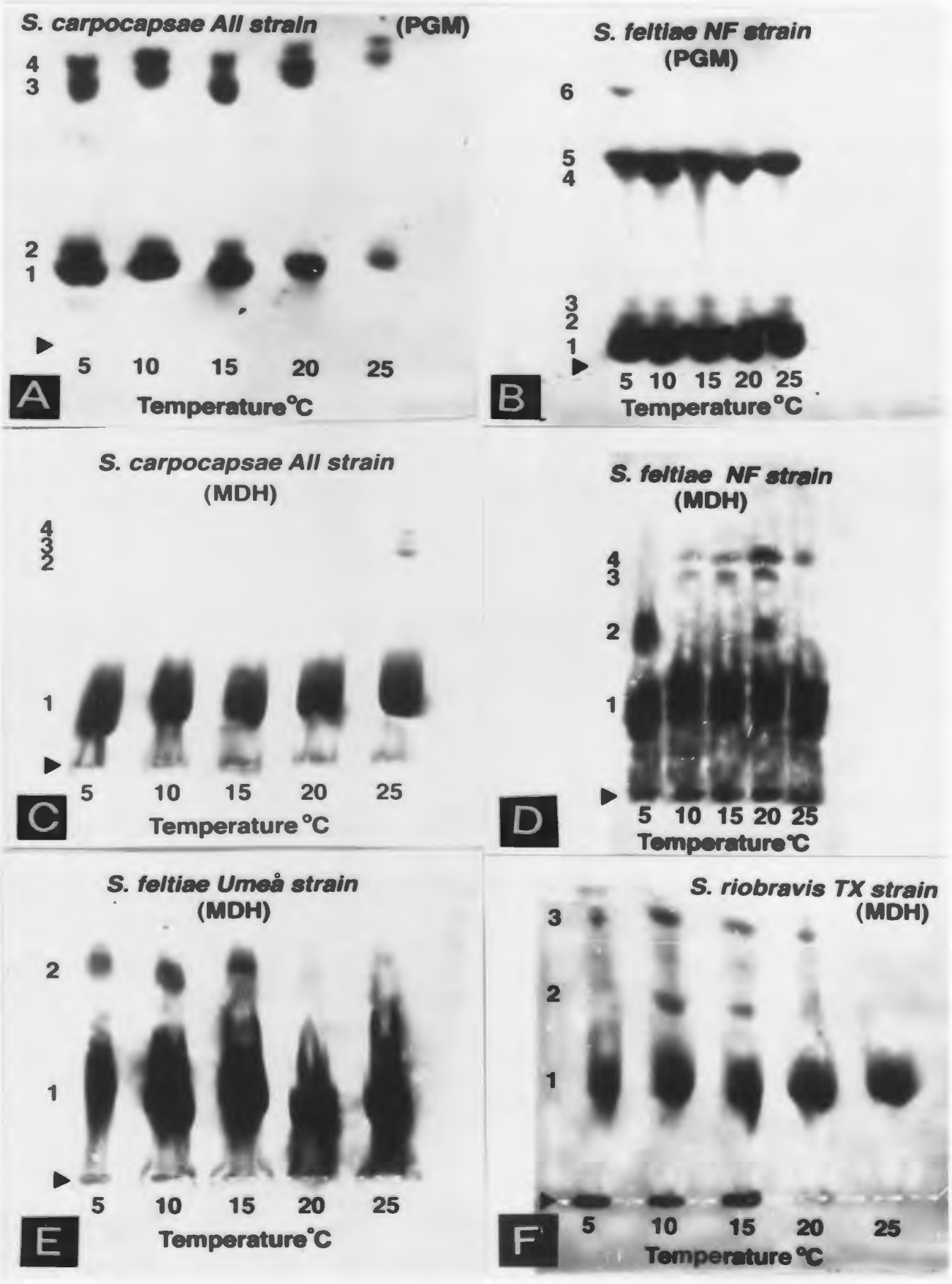

S. carpocapsae All strain (PGM)
4
3
2
1
5 10 15 20 25
A
Temperature°C
S. feltiae NF strain
(PGM)
6
5
4
3
2
1
5 10 15 20 25
B
Temperature°C
S. carpocapsae All strain
(MDH)
4
3
2
1
5 10 15 20 25
C
Temperature °C
S. feltiae NF strain
(MDH)
4
3
2
1
5 10 15 20 25
D
Temperature°C
S. feltiae Umeå strain
(MDH)
2
1
5 10 15 20 25
E
Temperature°C
S. riobravis TX strain
(MDH)
3
2
1
5 10 15 20 25
F
Temperature °C

Table 1. Mean electrophoretic mobility ($cm^2/sec./v$) of isoenzymes of four enzymes in four isolates of steinernematid nematodes recycled and stored at five temperature regimes.

# of[a] band	5 °C	10 °C	15 °C	20 °C	25 °C
			***Steinernema carpocapsae* All strain**		
			Glycerol-3-phosphate dehydrogenase (G3PDH)		
1.	2.39×10^{-4}	2.26×10^{-4}	2.48×10^{-4}	2.46×10^{-4}	2.46×10^{-4}
			Malate dehydrogenase (MDH)		
1.	7.45×10^{-5}	7.45×10^{-5}	7.45×10^{-5}	7.45×10^{-5}	7.45×10^{-5}
2.	-	-	-	-	2.67×10^{-4}
3.	-	-	-	-	2.82×10^{-4}
4.	-	-	-	-	3.04×10^{-4}
			Mannose-6-phosphate isomerase (MPI)		
1.	1.54×10^{-4}	1.62×10^{-4}	1.62×10^{-4}	1.65×10^{-4}	1.65×10^{-4}
2.	1.83×10^{-4}	1.98×10^{-4}	2.05×10^{-4}	2.05×10^{-4}	2.05×10^{-4}
			Phosphoglucomutase (PGM)		
1.	8.61×10^{-5}	8.81×10^{-5}	9.01×10^{-5}	8.81×10^{-5}	8.81×10^{-5}
2.	1.09×10^{-4}	1.15×10^{-4}	1.13×10^{-4}	-	-
3.	2.93×10^{-4}	2.93×10^{-4}	2.87×10^{-4}	2.93×10^{-4}	2.95×10^{-4}
4.	3.11×10^{-4}	3.11×10^{-4}	3.09×10^{-4}	3.15×10^{-4}	3.13×10^{-4}

Table 1 continued

# of[a] band	5 °C	10 °C	15 °C	20 °C	25 °C
			***S. feltiae* NF strain**		
			Glycerol-3-phosphate dehydrogenase (G3PDH)		
1.	1.93×10^{-4}	1.91×10^{-4}	1.89×10^{-4}	1.96×10^{-4}	1.93×10^{-4}
			Malate dehydrogenase (MDH)		
1.	1.22×10^{-4}	1.30×10^{-4}	1.30×10^{-4}	1.32×10^{-4}	1.32×10^{-4}
2.	1.67×10^{-4}	-	-	1.67×10^{-4}	-
3.	-	2.30×10^{-4}	2.32×10^{-4}	2.41×10^{-4}	-
4.	-	2.76×10^{-4}	2.76×10^{-4}	2.79×10^{-4}	2.79×10^{-4}
			Mannose-6-phosphate isomerase (MPI)		
1.	4.59×10^{-5}	-	-	-	-
2.	1.64×10^{-4}	1.64×10^{-4}	1.64×10^{-4}	1.64×10^{-4}	1.65×10^{-4}
3.	1.90×10^{-4}	1.90×10^{-4}	1.92×10^{-4}	1.88×10^{-4}	1.88×10^{-4}
4.	2.06×10^{-4}	2.06×10^{-4}	2.06×10^{-4}	2.04×10^{-4}	2.07×10^{-4}
5.	2.84×10^{-4}	-	-	3.04×10^{-4}	-
6.	3.51×10^{-4}				
			Phosphoglucomutase (PGM)		
1.	2.43×10^{-5}	2.43×10^{-5}	2.43×10^{-5}	2.43×10^{-5}	2.43×10^{-5}
2.	4.40×10^{-5}	4.46×10^{-5}	4.67×10^{-5}	4.67×10^{-5}	4.67×10^{-5}
3.	6.37×10^{-5}	6.37×10^{-5}	6.23×10^{-5}	6.23×10^{-5}	6.23×10^{-5}
4.	-	2.06×10^{-4}	-	-	-
5.	2.39×10^{-4}	2.31×10^{-4}	2.31×10^{-4}	2.31×10^{-4}	2.31×10^{-4}
6.	3.03×10^{-4}	-	-	-	-

Table 1 continued

# of[a] band	5 °C	10 °C	15 °C	20 °C	25 °C
			***S. feltiae* Umeå strain**		
			Glycerol-3-phosphate dehydrogenase (G3PDH)		
1.	1.98×10^{-4}	2.15×10^{-4}	2.16×10^{-4}	2.15×10^{-4}	2.15×10^{-4}
			Malate dehydrogenase (MDH)		
1.	1.56×10^{-4}	1.56×10^{-4}	1.58×10^{-4}	1.58×10^{-4}	1.58×10^{-4}
2.	3.21×10^{-4}	2.92×10^{-4}	2.92×10^{-4}	-	-
			Mannose-6-phosphate isomerase (MPI)		
1.	1.22×10^{-4}	1.22×10^{-4}	1.24×10^{-4}	1.26×10^{-4}	1.28×10^{-4}
2.	1.51×10^{-4}	1.43×10^{-4}	1.50×10^{-4}	1.58×10^{-4}	1.61×10^{-4}
3.	1.79×10^{-4}	1.83×10^{-4}	1.87×10^{-4}	1.87×10^{-4}	-
			Phosphoglucomutase (PGM)		
1.	2.60×10^{-4}	2.60×10^{-4}	2.60×10^{-4}	2.60×10^{-4}	2.60×10^{-4}
2.	2.73×10^{-4}	2.73×10^{-4}	2.73×10^{-4}	2.73×10^{-4}	2.73×10^{-4}

Table 1 continued

# of[a] band	5 °C	10 °C	15 °C	20 °C	25 °C
			***S. riobravis* TX strain**		
			Glycerol-3-phosphate dehydrogenase (G3PDH)		
1.	2.35 x 10^{-4}	2.35 x 10^{-4}	2.38 x 10^{-4}	2.38 x 10^{-4}	2.38 x 10^{-4}
			Malate dehydrogenase (MDH)		
1.	1.33 x 10^{-4}	1.39 x 10^{-4}	1.33 x 10^{-4}	1.29 x 10^{-4}	1.37 x 10^{-4}
2.	-	2.33 x 10^{-4}	2.33 x 10^{-4}	-	-
3.	2.67 x 10^{-4}	2.71 x 10^{-4}	2.58 x 10^{-4}	2.44 x 10^{-4}	-
			Mannose-6-phosphate isomerase (MPI)		
1.	1.85 x 10^{-4}	2.04 x 10^{-4}	2.00 x 10^{-4}	1.90 x 10^{-4}	1.93 x 10^{-4}
2.	2.14 x 10^{-4}	2.28 x 10^{-4}	2.24 x 10^{-4}	2.18 x 10^{-4}	2.20 x 10^{-4}
			Phosphoglucomutase (PGM)		
1.	1.08 x 10^{-4}	1.06 x 10^{-4}	1.04 x 10^{-4}	1.10 x 10^{-4}	1.08 x 10^{-4}
2.	2.22 x 10^{-4}	2.30 x 10^{-4}	2.28 x 10^{-4}	2.28 x 10^{-4}	2.34 x 10^{-4}

Values are the means of three replicate plates, each replicate representing a separate homogenate of infecive juveniles. [a]For each enzyme of each isolate, bands were numbered in increasing numerical order relative to the distance that they migrated cathodally from the origin: they do not necessarily correspond numerically among the isolates. Dashes indicate absence of bands.

CHAPTER 6

EFFECT OF TEMPERATURE ON THE ACTIVITIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND HEXOKINASE IN ENTOMOPATHOGENIC NEMATODES (NEMATODA: STEINERNEMATIDAE).

6.1. ABSTRACT

The kinetic properties of two metabolic enzymes, glucose-6-phosphate dehydrogenase and hexokinase, were studied in four entomopathogenic nematodes that had been recycled through wax moth (*Galleria mellonella*) larvae for two years at various temperatures: *Steinernema feltiae* NF strain, *Steinernema feltiae* Umeå strain, *Steinernema carpocapsae* All strain, *Steinernema riobravis* TX strain. The recycling temperatures influenced the activities of glucose-6-phosphate dehydrogenase and hexokinase in an adaptive fashion in all the isolates. At each assay temperature (5- 35°C), the maximum specific activity of both the enzymes was greater in the nematodes that had been recycled at lower temperatures than in those recycled at higher temperatures. In three enzyme - nematode isolate combinations, the lowest K_m values occurred in nematodes that had been recycled at the lower temperatures. However, such low K_m values did not correspond with the recycling temperatures.

6.2. INTRODUCTION

Steinernematid and heterorhabditid nematodes have potential in controlling populations of soil-inhabiting and otherwise cryptic insects (Klein, 1990). Free-living infective juveniles of these nematodes are found in the soil and are mutualistically associated with a species-specific bacterium (Akhurst and Boemare, 1990). Infective juveniles gain access to the hemocoel of insect hosts via natural openings, then each infective juvenile releases the mutualistic bacteria from its anus. The bacteria play an

important role in killing the insect host and providing a nutritional milieu conducive for nematode development (Poinar, 1990).

Although, in some instances, the field efficacy of entomopathogenic nematodes can be equivalent to insecticides (Georgis and Gaugler, 1991), their success in pest management is limited by several environmental factors (Kaya, 1990). Temperature is one of the most important factors that influence the survival, infectivity, reproduction and development of entomopathogenic nematodes (Griffin, 1993, Georgis and Gaugler, 1991; Grewal *et al.*, 1994; Mason and Hominick, 1995). Nevertheless, entomopathogenic nematodes have been reported from a wide variety of climatic regions (Amarasinghe *et al.*, 1994; Cabanillas *et al.*, 1994; Griffin *et al.* 1991; Jagdale *et al.*, 1996; Mràček and Webster, 1993).

In previous studies, it was determined that the capacities of steinernematids to tolerate temperature extremes may be modified by the temperature at which they are recycled (see Chapter 3). The mechanisms involved in such temperature adaptation included modifications in synthesis of isozymes (see Chapter 5) and degree of unsaturation of fatty acids (see Chapter 7), strategies commonly deployed by other groups of poikilotherms (Hazel, 1995; Hazel and Prosser, 1974; Johnston, 1983; Somero, 1995). Another key strategy deployed by poikilotherms for adapting to changing environmental temperatures is the capacities of several metabolic enzymes and enzymes associated with the nervous system to modify their structural and kinetic properties in accordance with the temperature regime (Baldwin, 1971; Hazel and Prosser, 1974; Hochachka and Somero,

1984: Sidell, 1983). However, no information is available on the effect of temperature on the kinetics of metabolic enzymes in nematodes.

The purpose of the present investigation was to determine whether there was a change in the activities of two enzymes: glucose-6-phosphate dehydrogenase, a key enzyme in the pentose- phosphate pathway, and hexokinase, crucial for glycolysis, in four strains of *Steinernema*, which had been recycled in the laboratory for prolonged periods of time at various temperature regimes.

6.3. MATERIALS AND METHODS

6.3.1. Sources of Nematodes

Steinernema carpocapsae All strain was provided by Plant Products Ltd., Brampton, Ontario, Canada; *S. riobravis* TX strain by Dr. H. E. Cabanillas, USDA, ARS, Crop Insects Research Unit, Weslaco, TX., U.S.A. *Steinernema feltiae* Umeå strain was provided by Dr. R. West, Canadian Forest Service (CFS), St. John's, NF, Canada from a stock colony that had been obtained initially from Biologic Biocontrol Products, Willow Hill, PA., U.S.A. *Steinernema feltiae* NF strain is a new strain (Jagdale *et al.*, 1996) that isolated in Summer 1993 from soil on an organic garden close to St. John's, NF, Canada, using *Galleria* bait traps (Woodring and Kaya, 1988).

6.3.2. Recycling temperature regimes

All nematode strains were recycled for two years (May, 1994 - May, 1996) by propagation through *G. mellonella* larvae (Woodring and Kaya, 1988; see Appendix I): *S. feltiae* Umeå strain at 10, 15, 20 and 25 °C; *S. feltiae* NF strain at 10, 15 and 20 °C; *S. carpocapsae* All strain at 15, 20 and 25 °C and *S. riobravis* TX strain at 20 and 25 °C.

6.3.3. Extraction of enzymes

Infective juveniles of each of the four isolates were transferred from the dilute formalin in the White traps (Woodring and Kaya, 1988) to separate 250- ml beakers containing distilled water, then allowed to settle (ca. 10 min). Using a Gilson$^{(R)}$ pipetman, infective juveniles were then transferred from the bottom of the beakers to Whatman no. 4 filter papers, so that they formed confined blobs on the papers. Samples of NF, Umeå, and All strains (500 mg wet weight) and TX strain (625 mg wet weight) for glucose-6-phosphate dehydrogenase activity and samples of All strain (500 mg wet weight), TX strain (625 mg wet weight) and Umeå strain (700 mg wet weight) for hexokinase activity were transferred into separate test tubes (ca. 15 ml). Each sample was then homogenized separately in 1 ml 0.09 M Tris-HCL buffer (pH 8.0) in a Potter-Elvehjem tissue grinder with a motor-driven pestle at room temperature (25°C). Each homogenate was transferred into a separate test tube containing 4 ml 0.09 M Tris-HCL buffer (pH 8.0), then samples were vortexed. The homogenate was centrifuged at 13,150 RPM for five minutes at room temperature (25°C), and the supernatant transferred to further test tubes and kept on ice

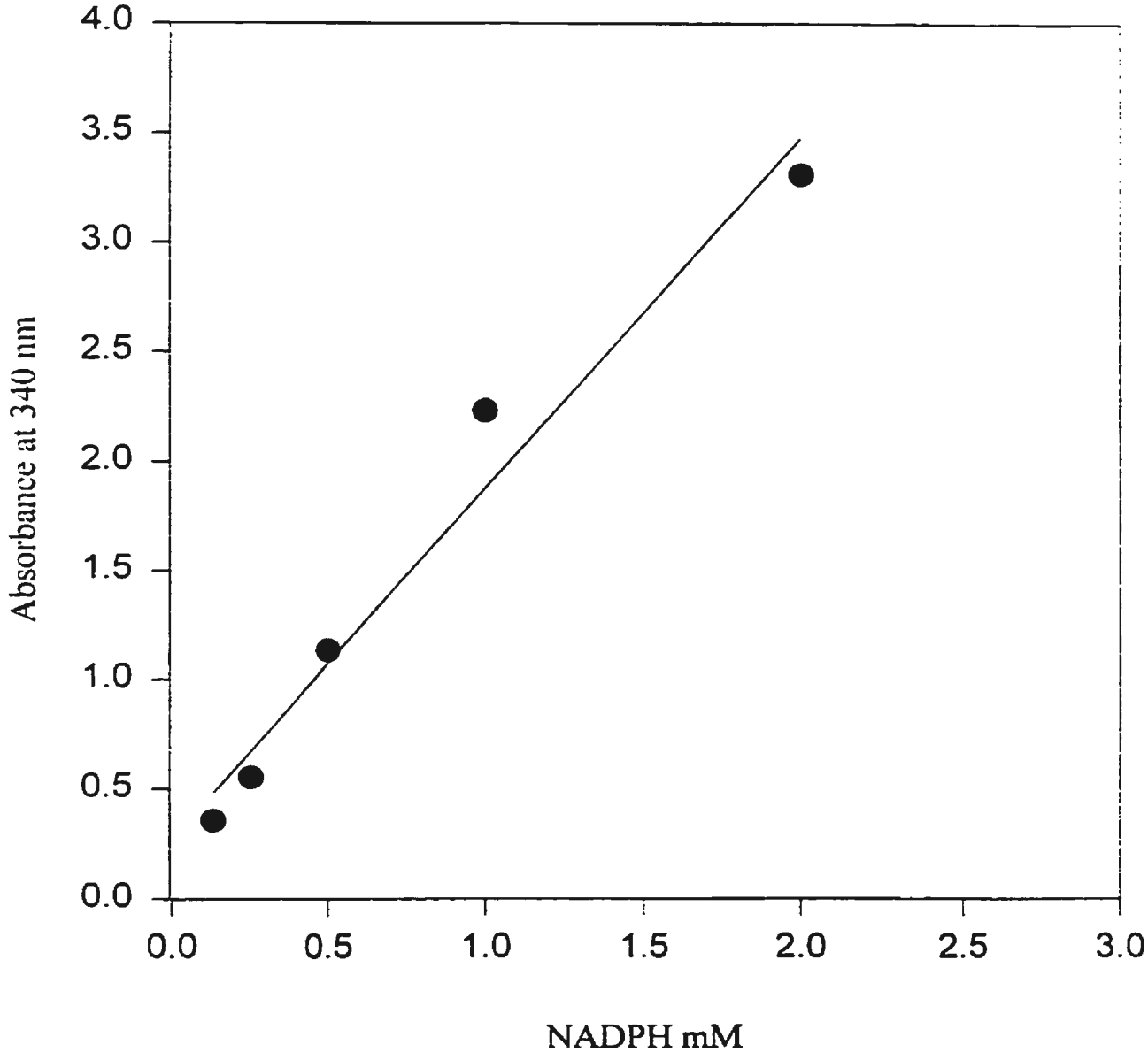
until used for determination of enzyme activities. Aliquots (200 µl) of the supernatant (enzyme extract) were used for determination of enzyme activity.

6.3.3.1. Enzyme assay for glucose-6-phosphate dehydrogenase activity

The enzyme assay, designed to measure production of NADPH (Fig, 1) was modified after Reimers *et al.* (1993). The reaction rates of glucose-6-phosphate dehydrogenase activity were determined by measuring an increase in absorbance at 340 nm with a Beckman DU(R)6 UV-Visible Spectrophotometer (Beckman Instruments Inc., Toronto, ON, Canada). The assays were performed following the formation of NADPH at four different assay temperatures, 5, 15, 25 and 35 °C. The cuvette temperature was controlled with a circulation water bath (Haake Mess-Technik GmbH u. Co., Karlsruhe, West Germany).

Three separate tubes of reaction mixture were prepared and each tube was considered as a separate replicate (n=3). The reaction mixture consisted of 400 µl 0.09 M Tris-HCL buffer (pH 8.0), 300 µl glucose-6-phosphate (40, 80, 160, 320 or 360 mM) as the substrate, 100 µl NADP (100 µl 1 mM NADP in 900 µl distilled water) as the coenzyme (concentrations given represent the final concentrations in the assay solution) and 200 µl nematode (enzyme) extract. The reaction was started by simultaneous addition of glucose-6-phosphate and NADP.

Fig. 1. NADPH (β- Nicotinamide adenine dinucleotide phosphate, reduced form) standard curve. Data are shown as NADPH concentration (mM) ploted against absobance at 340 nm.


4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Absorbance at 340 nm
0.0
0.5
1.0
1.5
2.0
2.5
3.0
NADPH mM

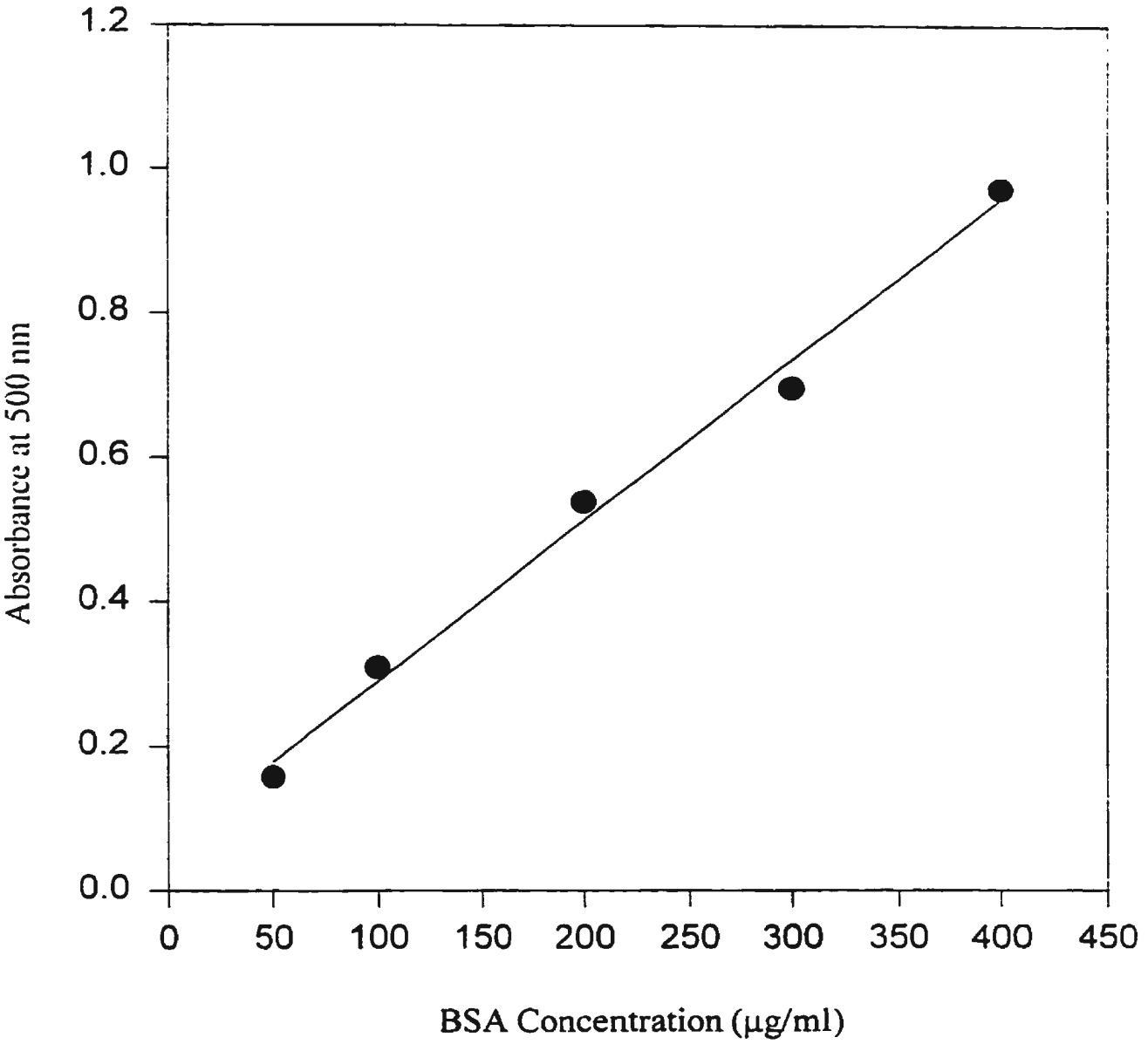
6.3.3.2. Enzyme assay for hexokinase activity

Hexokinase activity was assayed in only three isolates of *Steinernema* (All. Umeå and TX strains) using the method described by Liu and Rothstein (1976), with modifications. The principle of the method was the same as that for glucose-6-phosphate dehydrogenase, viz. an increase in absorbance at 340 nm due to production of NADPH (Fig. 1). The reaction mixture consisted of 250 µl 0.09 M Tris-HCL buffer (pH 8.0), 300 µl glucose (0.1, 0.2, 0.8, 2.5 or 10 mM) as the substrate, 100 µl NADP (2 mM) as the coenzyme, 100 µl ATP (7.5 mM), 100 µl $MgCl_2$ (40 mM), 50 µl glucose-6-phosphate dehydrogenase (50 units / 990 µl distilled water) as the coupling enzyme (concentrations given represent the final concentrations in the assay solution) and 200 µl nematode (enzyme) extract. The reaction was started by simultaneous addition of the coupling enzyme, glucose-6-phosphate dehydrogenase, and the nematode (enzyme) extract.

6.3.4. Protein assay

Protein was analyzed by the Lowry method (Lowry *et al.*, 1951), modified by Peterson (1977) using bovine serum albumin as the standard (Protein assay kit, Sigma Diagnostics(R), St. Louis, MO, USA); absorbance measurements were made at 500 nm (Fig. 2) with a Spectronic(R) Genesys™ 5 Spectrophotometer (Milton Roy Co., Rochester, NY, USA).

Fig. 2. Protein calibration curve. Bovine serum albumin was used as a standard (μg/ml). Absorbance were measured at 500 nm.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance at 500 nm
0
50
100
150
200
250
300
350
400
450
BSA Concentration (µg/ml)

6.3.5. Enzyme kinetics

Specific activities of both the enzymes were expressed as enzyme activity (units) per mg nematode protein. One unit is defined as the enzyme activity that converts 1 μmol of substrate in 1 min under the above conditions. Michaelis-Menten constants (K_m) and maximum specific activities for glucose-6-phosphate and glucose were determined from double reciprocal plots of reaction velocity vs substrate concentration (Lineweaver-Burk plots).

6.3.6. Statistical analysis

The data (log_{10} transformed) were analysed using one way ANOVA followed by Student- Newman- Keuls test (Jandel Corp$^{(R)}$. Sigma Stat, 1992) to compare the mean maximum specific activity (units/mg protein) and mean K_m values (mM) between the assay and recycling temperatures. Comparisons of specific activity data (log_{10} transformed) between recycling temperatures were also analysed by the General Linear Models Procedure for ANCOVA that incorporated a Duncan's multiple range test for the variable. This was done using Statistical Analysis System (SAS for Windows 6.10), a computer package (SAS Institute Inc., 1994). The level of significance was defined as $P< 0.05$.

6.4. RESULTS

The recycling temperatures influenced the kinetic properties of both glucose-6-phosphate dehydrogenase and hexokinase in all three species (four strains) of

entomopathogenic nematodes. Regardless of recycling temperature, the maximum specific activities of both the enzymes increased significantly as the assay temperature increased from 5 to 35 °C (Tables 1 and 3). However. at each assay temperature, the maximum activity of these enzymes was recorded from the nematodes that had been recycled at lower temperatures (Figs. 1A - 4A). Statistical comparisons of specific activity- assay temperature curves showed significant differences between recycling temperatures for both enzymes in all four isolates.

In three instances (*S. feltiae* NF strain and *S. carpocapsae* All strain: glucose-6-phosphate dehydrogenase; *S. riobravis* TX strain: hexokinase), enzyme substrates had K_m values (Tables 2 and 4) that were correlated with recycling temperatures, such that the lowest K_m values occurred in the nematodes that had been recycled at the colder temperatures (Figs. 2B- 4B). However, the size of the lowest K_m value (Table 2) for glucose-6-phosphate dehydrogenase substrate was unaffected by recycling temperatures (20, 25 °C) in *S. riobravis* (Fig. 4B) and no clear cut recycling temperature correlations were evident with respect to the size of the lowest K_m values for the hexokinase substrate of *S. carpocapsae* All strain (Fig. 3B) and substrates of both enzymes of the Umeå strain of *S. feltiae* (Fig. 1B).

The lowest K_m value (Tables 2 and 4) for substrates of both glucose-6-phosphate dehydrogenase and hexokinase occurred at ≥15 °C assay temperatures (Figs. 1B- 4B), with the exception of the hexokinase substrate of *S. feltiae* Umeå strain that had been maintained at 10 °C, in which K_m values (Table 4) were independent of assay temperature

(Fig. 1B). There were no obvious situations in which there was a clearly defined correlation between the recycling temperature and the temperature at which the enzyme substrate for a nematode isolate displayed its lowest K_m value.

6.5. DISCUSSION

This study has shown that the maximum specific activities of two metabolic enzymes in four isolates of entomopathogenic nematodes were altered in an adaptive fashion by the temperature regimes at which recycling was carried out. The enzymes of nematodes that had been recycled at colder temperatures permitted more rapid substrate catalysis at all assay temperatures than those that had been recycled at warmer temperatures. This compensatory response to the otherwise decelerating effect of declining temperature was displayed by all nematode isolates, regardless of their sites of origin, over the temperature ranges where recycling was possible. In the case of the boreally adapted Umeå and NF strains of *S. feltiae*, first isolated from northern Sweden (Pye and Pye, 1985) and Newfoundland, Canada (Jagdale *et al.*, 1996) respectively, recycling was carried out over the range 10-25 °C. The warm temperate *S. carpocapsae* All strain, first isolated from Georgia, U.S.A. (Poinar, 1979), was recycled at 15-25 °C, while the subtropical *S. riobravis* TX strain, from southern Texas, U.S.A. (Cabanillas *et al.*, 1994), was recycled at 20- 25 °C.

The effect of recycling temperature on the apparent Michaelis-Menten constant (K_m) values varied according to the nematode strain and enzyme assayed. There were

three instances in which enzyme substrates had lowest K_m values that appeared to be adaptively correlated with recycling temperatures. such that the lowest K_m values (i.e. highest enzyme - substrate affinities) occurred in the nematodes that had been recycled at the colder temperatures. However. in contrast to what has been found to be the case for enzyme systems subjected to acclimation in many teleosts (Baldwin, 1971; Baldwin and Hochachka. 1970; Ozernyuk *et al.*. 1994). the assay temperatures at which the K_m values were lowest did not correspond with the recycling temperatures. The current findings are similar to those of Vetter (1995), who showed that the size of the K_m value for acetyl-coenzyme A and oxaloacetate. the substrates of citrate synthase, were changed adaptively by acclimation temperatures in crustacea, although the temperatures at which the lowest K_m value occurred were not affected. In the present study, glucose-6-phosphate dehydrogenase in cold adapted *S. feltiae* NF strain would be expected to operate no more efficiently at temperatures ≤ 15°C than in warm adapted nematodes, because the lowest K_m value occurred at 25 °C. For the same reason, the efficiency of this enzyme system was more efficient in *S. carpocapsae* All strain that had been recycled at 15 and 20 °C than in those recycled at 25 °C, but the difference between these groups became progressively less as the assay temperature decreased from 25 °C. Only in the case of the hexokinase substrate for *S. riobravis* TX strain did it appear that the enzyme was adaptively altered to allow substantially improved efficiency in nematodes that had been recycled at the lower temperature (20 cf. 25 °C).

Thus, it would appear that, for the most part, the adaptive changes in enzyme-

specific activities resulting from recycling at colder temperatures are not the result of conformational changes in the enzymes that would bring about alterations in enzyme - substrate affinities. It may be that changes in enzyme concentration, a strategy that is best suited for long- term temperature adaptation (Hochachka and Somero, 1984), were induced by the temperatures at which the nematodes were recycled. Indeed, since the nematodes were propagated, rather than simply acclimated, changes in enzyme- specific activities, possibly involving concentration effects, could be of genetic rather than purely environmental origin and the result of artificial selection.

Whether the K_m- assay temperature curves are indicative of different isozymes synthesized at the various recycling temperatures or to allosteric modulators causing conformational changes in the enzyme (Stryer, 1988) is not known. At least in the case of hexokinase in *S. riobravis* TX strain, the two curves determined from assays of the nematodes recycled at 20 and 25°C are sufficiently distinct to suggest that the first possibility (i. e. synthesis of new isozymes) may be the case. In a previous study, we have shown that isozymes of other enzymes were synthesized at cold temperatures in the NF and Umeå strains of *S. feltiae*, while *S. carpocapsae* All synthesized both cold and warm adapted isozymes in response to recycling temperature (see Chapter 5).

The capacity of these nematodes to compensate for long term changes in environmental temperature through alterations in enzyme specific activities and synthesis of isozymes is augmented by adaptive changes in the degree of unsaturation of fatty acids in phospholipids and storage lipids (see Chapter 7). Physiological adjustments to recycling

temperatures are accompanied by adaptive changes in the ability of the nematodes to survive at temperature extremes (see Chapter 3). A full understanding of the abilities of entomopathogenic nematodes to adapt to varying recycling regimes and the underlying physiological mechanisms involved should prove helpful in their commercial mass production for pest management.

Fig. 3. Effect of different recycling temperatures (RT) on the maximum specific activities (A) and K_m values (B) for substrates of glucose-6-phosphate dehydrogenase and hexokinase extracted from *Steinernema feltiae* Umeå strain at various assay temperatures. Each point represents the mean ± SE of three replicates.

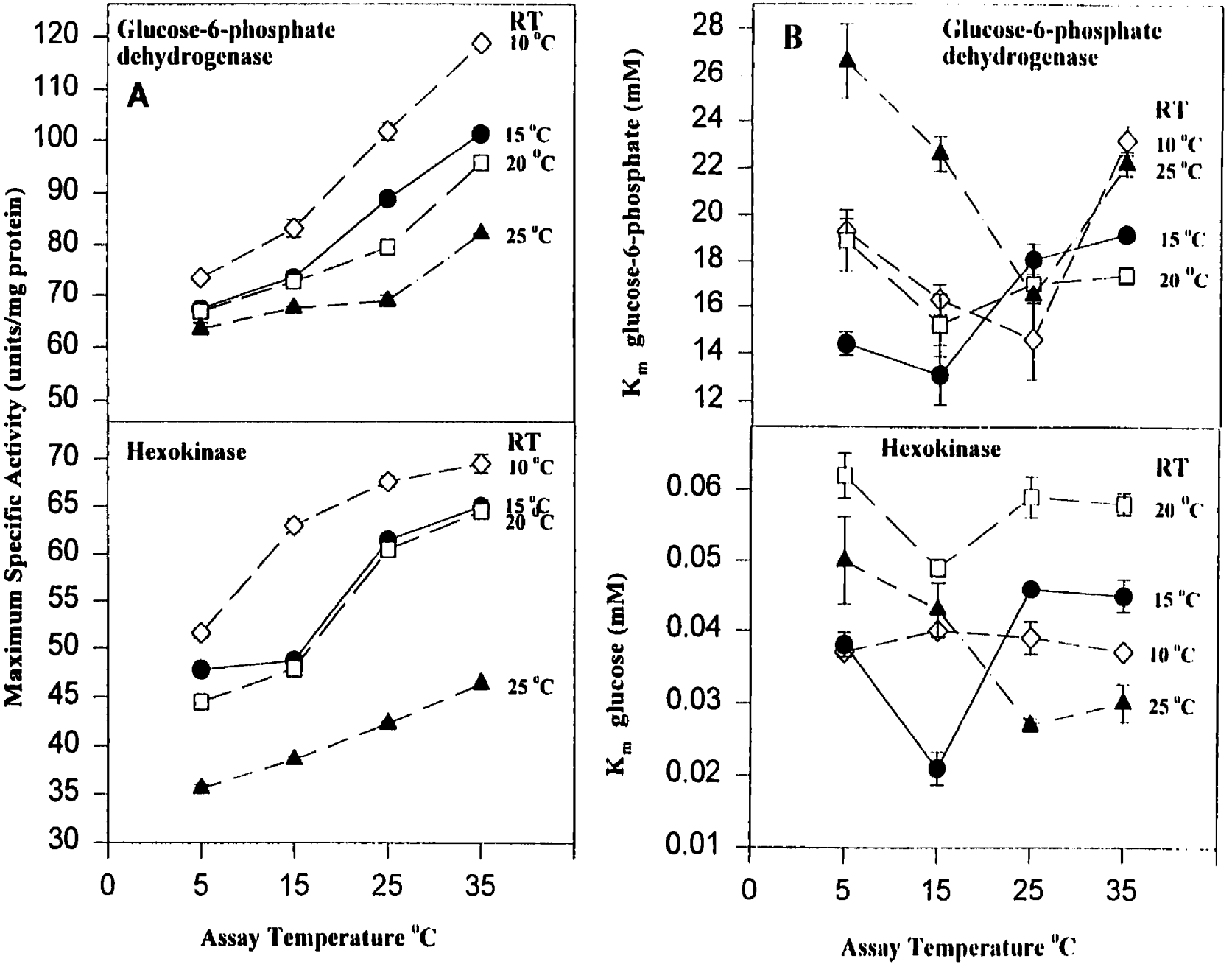

A
Glucose-6-phosphate dehydrogenase
RT
10 °C
15 °C
20 °C
25 °C
Hexokinase
RT
10 °C
15 °C
20 °C
25 °C
Maximum Specific Activity (units/mg protein)
Assay Temperature °C
B
Glucose-6-phosphate dehydrogenase
RT
10 °C
25 °C
15 °C
20 °C
Hexokinase
RT
20 °C
15 °C
10 °C
25 °C
K_m glucose-6-phosphate (mM)
K_m glucose (mM)
Assay Temperature °C

Fig. 4. Effect of different recycling temperatures (RT) on the maximum specific activities (A) and K_m values (B) for substrates of glucose-6-phosphate dehydrogenase and hexokinase extracted from *Steinernema feltiae* NF strain at various assay temperatures. Each point represents the mean ± SE of three replicates.

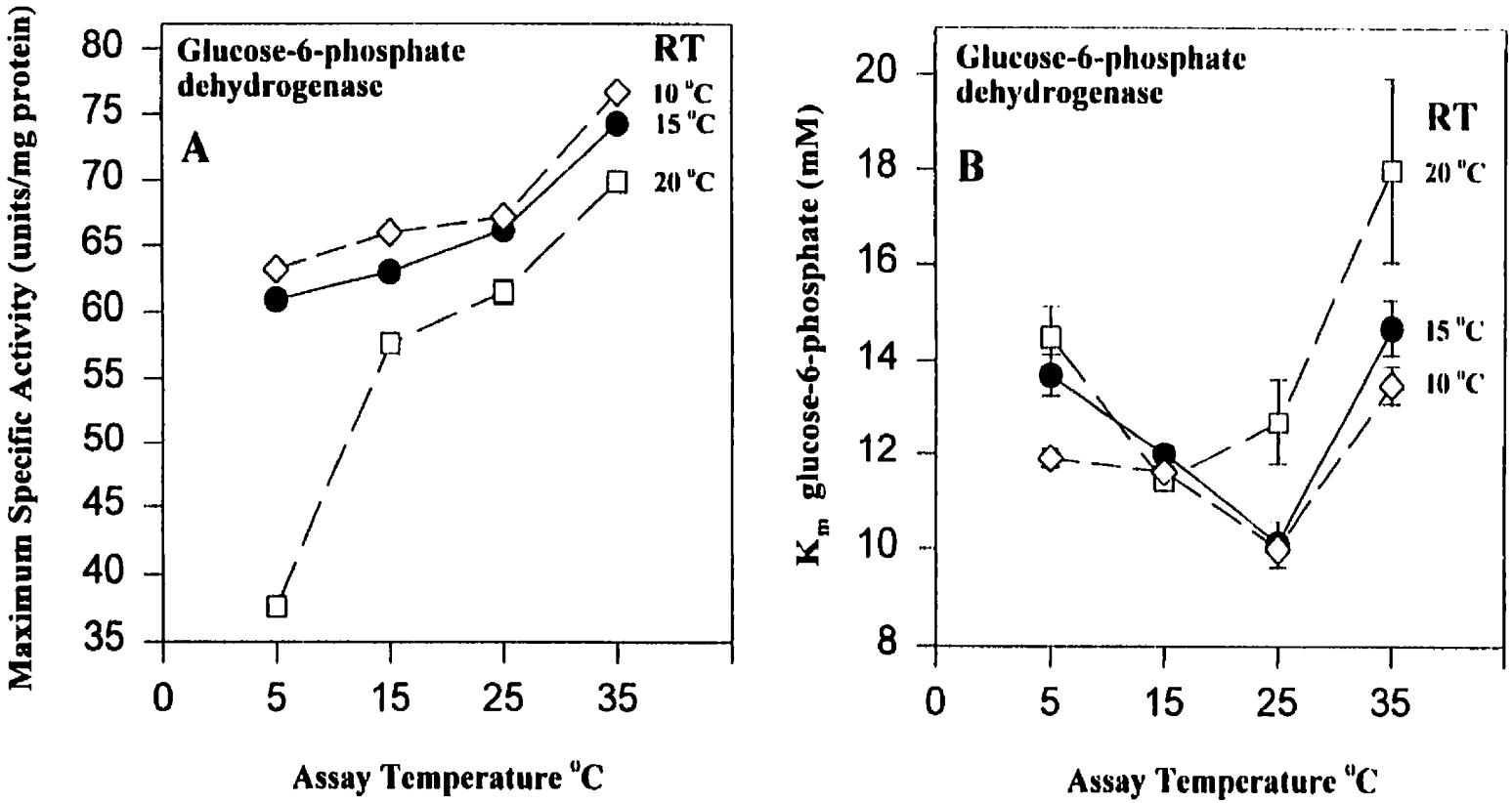

Maximum Specific Activity (units/mg protein)
80
75
70
65
60
55
50
45
40
35
Glucose-6-phosphate dehydrogenase
A
RT
10 °C
15 °C
20 °C
0
5
15
25
35
Assay Temperature °C
K_m glucose-6-phosphate (mM)
20
18
16
14
12
10
8
Glucose-6-phosphate dehydrogenase
B
RT
20 °C
15 °C
10 °C
0
5
15
25
35
Assay Temperature °C

Fig. 5. Effect of different recycling temperatures (RT) on the maximum specific activities (A) and K_m values (B) for substrates of glucose-6-phosphate dehydrogenase and hexokinase extracted from *Steinernema carpocapsae* All strain at various assay temperatures. Each point represents the mean ± SE of three replicates.

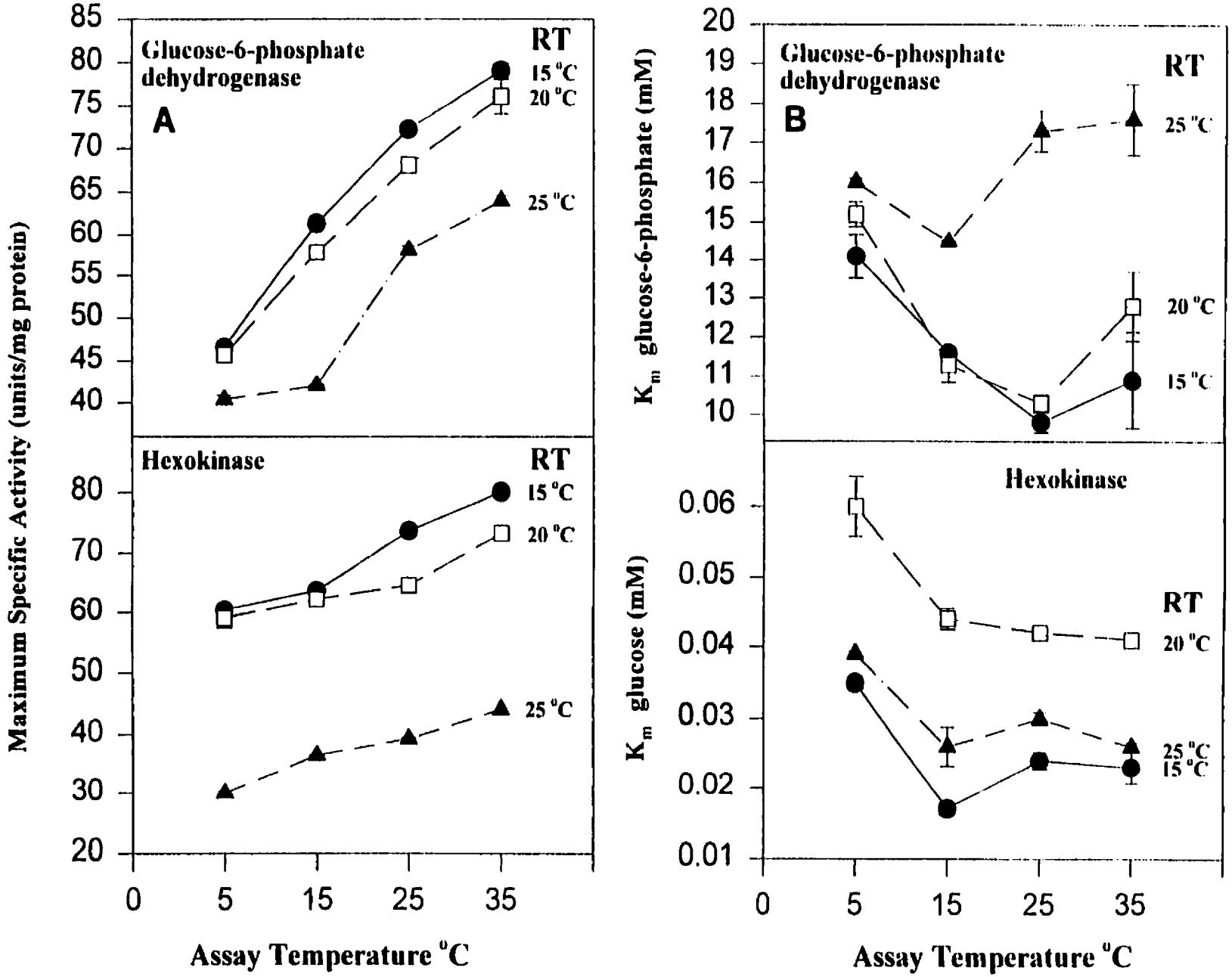

Maximum Specific Activity (units/mg protein)
Glucose-6-phosphate dehydrogenase
A
RT
15 °C
20 °C
25 °C
Hexokinase
RT
15 °C
20 °C
25 °C
Assay Temperature °C
K_m glucose-6-phosphate (mM)
Glucose-6-phosphate dehydrogenase
B
RT
25 °C
20 °C
15 °C
K_m glucose (mM)
Hexokinase
RT
20 °C
25 °C
15 °C
Assay Temperature °C

Fig. 6. Effect of different recycling temperatures (RT) on the maximum specific activities (A) and K_m values (B) for substrates of glucose-6-phosphate dehydrogenase and hexokinase extracted from *Steinernema riobravis* TX strain at various assay temperatures. Each point represents the mean ± SE of three replicates.

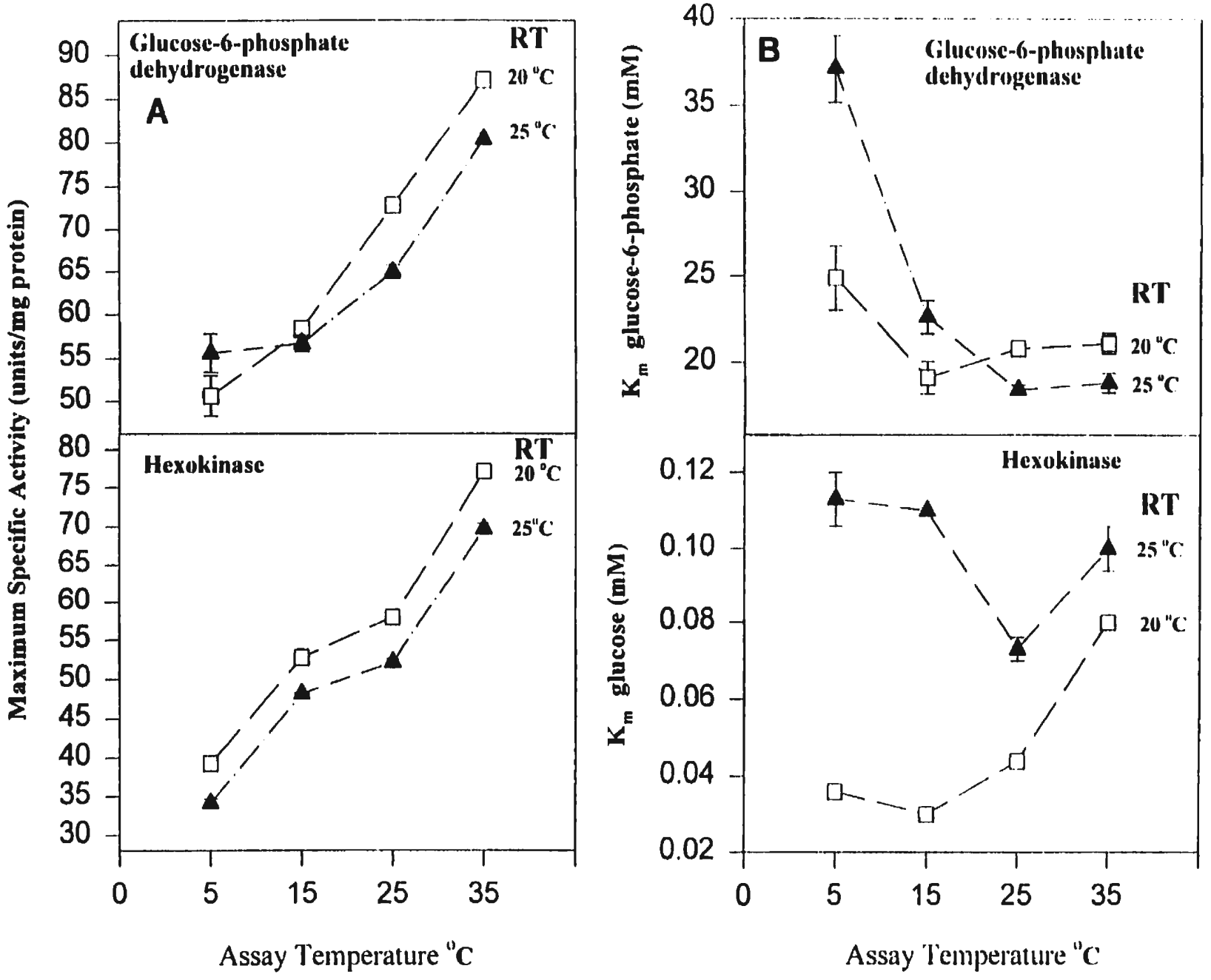

Maximum Specific Activity (units/mg protein)
Glucose-6-phosphate dehydrogenase
A
RT
20 °C
25 °C
Hexokinase
RT
20 °C
25°C
Assay Temperature °C
K_m glucose-6-phosphate (mM)
B
Glucose-6-phosphate dehydrogenase
RT
20 °C
25 °C
K_m glucose (mM)
Hexokinase
RT
25 °C
20 °C
Assay Temperature °C

Table 1. Maximum specific activity of glucose-6 phosphate dehydrogenase extracted from infective juveniles of entomopathogenic nematodes maintained at various temperatures.

Assay temperature (°C)	Maintenance Temperature (°C) 10	15	20	25
		Steinernema feltiae Umeå strain		
5	73.5 ± 0.3^{aA}	67.3 ± 0.2^{aB}	66.8 ± 0.6^{aC}	63.5 ± 1.1^{aD}
15	83.2 ± 1.7^{bA}	73.6 ± 1.2^{bB}	72.7 ± 0.9^{bC}	67.5 ± 0.3^{aD}
25	101.7 ± 1.7^{cA}	88.9 ± 0.5^{cB}	79.6 ± 1.3^{cC}	68.9 ± 1.1^{bD}
35	118.8 ± 1.1^{dA}	101.2 ± 1.1^{dB}	95.7 ± 0.3^{dC}	82.2 ± 0.1^{cD}
		Steinernema feltiae NF strain		
5	63.2 ± 0.1^{aA}	60.9 ± 0.6^{aA}	37.6 ± 0.4^{aB}	-
15	66.0 ± 0.1^{bA}	63.0 ± 0.1^{bA}	57.7 ± 0.1^{bB}	-
25	67.2 ± 0.3^{cA}	66.2 ± 0.3^{cA}	61.5 ± 0.9^{cB}	-
35	76.7 ± 0.2^{dA}	74.3 ± 0.5^{dA}	69.9 ± 0.8^{dB}	-
		Steinernema carpocapsae All strain		
5	-	46.6 ± 0.3^{aA}	45.7 ± 0.0^{aB}	40.4 ± 0.5^{aC}
15	-	61.4 ± 0.2^{bA}	57.9 ± 0.2^{bB}	42.1 ± 0.1^{bC}
25	-	72.3 ± 0.5^{cA}	68.1 ± 0.2^{cB}	58.2 ± 0.5^{cC}
35	-	79.1 ± 0.8^{dA}	76.1 ± 2.0^{dB}	64.0 ± 0.6^{dC}
		Steinernema riobravis TX strain		
5	-	-	50.6 ± 2.4^{aA}	55.6 ± 2.2^{aB}
15	-	-	58.3 ± 0.6^{bA}	56.7 ± 0.9^{aB}
25	-	-	72.8 ± 0.5^{cA}	65.0 ± 0.8^{bB}
35	-	-	87.1 ± 0.6^{dA}	80.4 ± 0.7^{cB}

Values are expressed as specific activity (units/mg protein) and are the means ± SE of three replicates. Means with the same upper case letter (across the columns) and means with the same lowercase letter (down the single column) are not significantly different ($P > 0.05$) by Student- Newman- Keuls test.

Table 2. Km values for glucose-6 phosphate dehydrogenase from infective juveniles of entomopathogenic nematodes maintained at various temperatures.

Assay temperature (°C)	Maintenance Temperature (°C) 10	15	20	25
		Steinernema feltiae Umeå strain		
5	19.3 ± 0.5^{aA}	14.4 ± 0.5^{aB}	18.9 ± 1.3^{aA}	26.6 ± 1.5^{aC}
15	16.3 ± 0.6^{abA}	13.1 ± 1.2^{aA}	15.2 ± 1.3^{aA}	22.6 ± 0.7^{bB}
25	14.6 ± 1.6^{bA}	18.1 ± 0.6^{bA}	17.0 ± 0.8^{aA}	16.5 ± 0.2^{cA}
35	23.2 ± 0.6^{cA}	19.2 ± 0.2^{bB}	17.4 ± 0.7^{aC}	22.2 ± 0.5^{bdA}
		Steinernema feltiae NF strain		
5	11.9 ± 0.2^{aA}	13.7 ± 0.4^{aB}	14.5 ± 0.6^{aB}	-
15	11.6 ± 0.1^{bA}	12.0 ± 0.2^{bA}	11.4 ± 0.1^{bA}	-
25	10.0 ± 0.0^{cA}	10.1 ± 0.5^{cA}	12.7 ± 0.9^{bB}	-
35	13.5 ± 0.4^{dA}	14.7 ± 0.6^{adA}	18.0 ± 1.9^{acA}	-
		Steinernema carpocapsae All strain		
5	-	14.1 ± 0.6^{aA}	15.2 ± 0.3^{aB}	16.0 ± 0.1^{aB}
15	-	11.6 ± 0.1^{bA}	11.3 ± 0.4^{bA}	14.5 ± 0.0^{aB}
25	-	9.8 ± 0.2^{bA}	10.3 ± 0.2^{cbA}	17.3 ± 0.5^{aB}
35	-	10.9 ± 1.2^{bA}	12.8 ± 0.8^{bdA}	17.6 ± 0.9^{aB}
		Steinernema riobravis TX strain		
5	-	-	24.9 ± 1.9^{aA}	37.1 ± 2.7^{aA}
15	-	-	19.1 ± 0.9^{bA}	22.6 ± 0.9^{bA}
25	-	-	20.8 ± 0.2^{abA}	18.4 ± 0.9^{bA}
35	-	-	21.1 ± 0.6^{abA}	18.8 ± 0.2^{bB}

Values are expressed as Mmoles of the substrate and are the means ± SE of three replicates. Means with the same upper case letter (across the columns) and means with the same lowercase letter (down the single column) are not significantly different ($P > 0.05$) by Student-Newman- Keuls test.

Table 3. Maximum specific activity of hexokinase extracted from infective juveniles of entomopathogenic nematodes maintained at various temperatures.

Assay temperature (°C)	Maintenance Temperature (°C) 10	15	20	25
		Steinernema feltiae Umeå strain		
5	51.6 ± 0.5^{a,A}	47.8 ± 0.4^{aB}	44.5 ± 0.7^{aC}	35.6 ± 0.4^{aD}
15	63.0 ± 0.7^{b,A}	48.7 ± 0.6^{aB}	47.9 ± 0.3^{bC}	38.6 ± 0.3^{bD}
25	67.6 ± 0.6^{c,A}	61.5 ± 0.4^{bB}	60.5 ± 0.3^{cC}	42.3 ± 0.2^{cD}
35	69.5 ± 1.0^{d,A}	65.0 ± 0.7^{cB}	64.5 ± 0.3^{dC}	46.4 ± 0.3^{dD}
		Steinernema carpocapsae All strain		
5	-	60.5 ± 0.3^{aA}	59.1 ± 1.6^{aB}	30.0 ± 0.3^{aC}
15	-	63.8 ± 0.3^{b,A}	62.3 ± 0.3^{bB}	36.5 ± 0.1^{bC}
25	-	73.7 ± 0.5^{c,A}	64.7 ± 1.0^{bB}	39.3 ± 0.2^{cC}
35	-	80.1 ± 0.8^{dA}	73.3 ± 0.1^{cB}	44.1 ± 0.0^{dC}
		Steinernema riobravis TX strain		
5	-	-	39.3 ± 0.3^{aA}	34.2 ± 0.5^{aB}
15	-	-	52.8 ± 0.2^{bA}	48.2 ± 0.1^{bB}
25	-	-	57.9 ± 0.8^{cA}	52.0 ± 0.4^{cB}
35	-	-	77.1 ± 0.8^{dA}	69.7 ± 0.7^{dB}

Values are expressed as specific activity (units/mg protein) and are the means ± SE of three replicates. Means with the same upper case letter (across the columns) and means with the same lowercase letter (down the single column) are not significantly different ($P > 0.05$) by Student- Newman- Keuls test.

Table 4. Km values for hexokinase from infective juveniles of entomopathogenic nematodes maintained at various temperatures.

Assay temperature (°C)	Maintenance Temperature (°C) 10	15	20	25
		Steinernema feltiae Umeå strain		
5	0.037 ± 0^{aA}	0.038 ± 0^{aA}	0.062 ± 0^{aB}	0.050 ± 0^{aC}
15	0.040 ± 0^{aA}	0.021 ± 0^{bB}	0.049 ± 0^{bA}	0.043 ± 0^{aA}
25	0.039 ± 0^{aA}	0.046 ± 0^{cB}	0.059 ± 0^{cC}	0.027 ± 0^{bD}
35	0.037 ± 0^{aA}	0.045 ± 0^{cB}	0.058 ± 0^{bcC}	0.030 ± 0^{bD}
		Steinernema carpocapsae All strain		
5	-	0.035 ± 0^{aA}	0.060 ± 0^{aB}	0.039 ± 0^{aC}
15	-	0.017 ± 0^{bA}	0.044 ± 0^{bB}	0.026 ± 0^{bC}
25	-	0.024 ± 0^{cA}	0.042 ± 0^{bB}	0.030 ± 0^{bC}
35	-	0.023 ± 0^{cA}	0.041 ± 0^{bB}	0.026 ± 0^{bA}
		Steinernema riobravis TX strain		
5	-	-	0.036 ± 0^{aA}	0.113 ± 0^{aB}
15	-	-	0.030 ± 0^{bA}	0.110 ± 0^{aB}
25	-	-	0.044 ± 0^{cA}	0.073 ± 0^{bB}
35	-	-	0.080 ± 0^{dA}	0.100 ± 0^{aB}

Values are expressed as Mmoles of the substrate and are the means ± SE of three replicates. Means with the same upper case letter (across the columns) and means with the same lowercase letter (down the single column) are not significantly different ($P > 0.05$) by Student- Newman- Keuls test.

CHAPTER 7

EFFECT OF TEMPERATURE ON THE COMPOSITION OF FATTY ACIDS IN TOTAL LIPIDS AND PHOSPHOLIPIDS OF ENTOMOPATHOGENIC NEMATODES

7.1. ABSTRACT

Gas liquid chromatography was used to determine the composition of fatty acids in total lipids and phospholipids of the entomopathogenic nematodes, *Steinernema feltiae* Umeå strain, *S. carpocapsae* All strain, *S. riobravis* TX strain and *S. feltiae* NF strain that had been recycled or stored at 5, 10, 15, 20 and 25°C. In all nematode isolates, the unsaturation indices of total lipids and phospholipids increased as recycling or storage temperatures decreased. This was due to an increase in polyunsaturated fatty acids with a concomitant decline in the proportion of saturated fatty acids, especially palmitic (16:0) and/or stearic (18:0) acids. The increase in polyunsaturated fatty acids at reduced temperatures was attributable to significantly greater percentages of linoleic acid (18:2) in total lipids and phospholipids. In all except *S. riobravis*, this was augmented by increased proportions of eicosapenic acid (20:5*w*3) at 5°C.

7.2. INTRODUCTION

Entomopathogenic nematodes (f. Steinernematidae and Heterorhabditidae) are being commercially produced for use in insect pest management (Smart, 1995). The soil dwelling infective juveniles of these nematodes infect susceptible insect species via the host's natural openings, then kill the host by releasing a mutualistic bacterium; nematode development and reproduction then occur within the host cadaver (Poinar, 1990) Among a plethora of factors known to influence the life cycle and field efficacy of these nematodes (Kaya, 1990), temperature affects their reproduction, development, dispersal

and infectivity (Kaya, 1977; Grewal *et al.*, 1994; Mason and Hominick, 1995; Griffin, 1993, 1996; Steiner, 1996).

Little is known about the physiological mechanisms involved in temperature adaptation of the nematodes. Many poikilothermic animals adapt to changing environmental temperatures by modifying the degree of unsaturation of their lipids. At low ambient temperatures, the proportion of unsaturated: saturated fatty acids increases in phospholipids to lower the transition temperature and thereby maintain cell membrane fluidity and normal cellular functions (Hazel, 1995; Hazel and Prosser, 1974; Hazel and Williams, 1990; Sinensky, 1974). The degree of unsaturation of triacylglycerols was found to be greater in the boreally- adapted mermithid nematode, *Neomesomermis flumenalis*, than in the tropical mermithid, *Romanomermis culicivorax*, a feature of adaptive significance in permitting maintenance of physiological state and accessibility of storage reserves to enzymes at cold temperatures (Gordon *et al.*, 1979). It has also been suggested that the synthesis of unsaturated fatty acids in the plant parasitic nematode, *Globodera rostochiensis*, may constitute a low-temperature adaptation mechanism (Gibson *et al.*, 1995). In the entomopathogenic nematode, *Steinernema carpocapsae*, decreasing the growth temperature from 25 to 18°C caused an increase in the unsaturation of fatty acids in the phospholipids, which in turn caused an increase in the membrane fluidity (Fodor *et al.*, 1994).

The commercialization of entomopathogenic nematodes involves continual recycling, so it is important to determine the degree to which their capacities for

temperature tolerance and underlying physiological mechanisms are affected by the recycling temperature regimes. It has been observed that the thermal tolerance ranges of four strains of *Steinernema* were modified by the temperature at which they were recycled (see Chapter 3) and that physiological manifestations of the changes in tolerance included changes in the synthesis of isozymes of several metabolic enzymes (see Chapter 5). The purpose of the present investigation was to determine whether there was a change in the composition of fatty acids in total lipids and phospholipids of these same isolates of *Steinernema*, which had been recycled or stored in the laboratory for prolonged periods of time at various temperature regimes.

7.3. MATERIALS AND METHODS

7.3.1. Sources of nematodes

Steinernema carpocapsae All strain was provided by Plant Products Ltd., Brampton, Ontario, Canada; *S. riobravis* TX strain by Dr. H. E. Cabanillas, USDA, ARS, Crop Insects Research Unit, Weslaco, TX., U.S.A. *Steinernema feltiae* Umeå strain was provided by Dr. R. West, Canadian Forest Service, St. John's, NF, Canada from a stock colony that had been obtained initially from Biologic Biocontrol Products, Willow Hill, PA., U.S.A. *Steinernema feltiae* NF strain is a new strain (Jagdale *et al.*, 1996) that we isolated in Summer 1994 from soil on a farm site close to St. John's, NF, Canada, using *Galleria* bait traps (Woodring and Kaya, 1988).

7.3.2. Recycling/storage temperature regimes

All nematode strains were recycled for two years (May. 1994 - May, 1996) by propagation through *Galleria mellonella* larvae (Woodring and Kaya, 1988; see Appendix I): NF and Umeå strains of *S. feltiae* at 10, 15, 20 and 25 °C; *S. carpocapsae* All strain at 15, 20 and 25 °C and *S. riobravis* TX strain at 20 and 25 °C. Infective juveniles of these nematodes were collected from their lowest recycling temperature regimes (*S. feltiae* from 10 °C, *S. carpocapsae* from 15 °C, *S. riobravis* from 20 °C) and stored in tissue culture bottles (600 ml) for three weeks at temperatures where recycling of isolates was not possible (*S. feltiae* at 5 °C, *S. carpocapsae* at 5 and 10 °C, *S. riobravis* at 5, 10 and 15 °C).

7.3.3. Extraction of lipids

Freshly emerged (0- 48 hour old) infective juveniles of each of the four isolates were transferred from the dilute formalin in the White traps (Woodring and Kaya, 1988) to separate 250 ml beakers containing distilled water, rinsed twice with distilled water (Gordon *et al.* 1996), then allowed to settle (ca. 10 min). Using a Gilson$^{(R)}$ pipetman, infective juveniles were transferred from the bottom of the beakers to Whatman no. 4 filter papers, so that they formed confined blobs on the papers. Three different samples of each isolate that had been recycling or stored at the specified temperature regimes (each sample 50 and 100 mg wet weight for total lipid and phospholipid analysis, respectively) were transferred into separate polypropylene microcentrifuge tubes (1.5 ml),

then frozen (-20 °C) overnight. The samples were then freeze- dried for 24 hours in a Labconco(R) freeze dry system, Lypho-lock 6 (Labconco Corp., Kansas City, MO, USA) and stored at -20 °C until used for analysis of fatty acids. Each tube was considered as a separate replicate (n= 3).

7.2.3.1. Analysis of total lipid fatty acids

Lipid fatty acid composition was determined using gas liquid chromatography (GLC). Lipids were extracted from infective juveniles using the method described by Bligh and Dyer (1959), with some modifications. Freeze- dried samples were homogenized in 200 µl methanol containing a few crystals of hydroquinone (antioxidant agent) in a Potter-Elvehjem tissue grinder with a motor-driven pestle. Each homogenate was then transferred into a separate polypropylene microcentrifuge tube (1.5 ml), then 250 µl distilled water, 250 µl chloroform and 550 µl methanol added; samples were vortexed and incubated at 4 °C for one hour. The homogenate was centrifuged at 13,150 RPM for 2 minutes at room temperature (25 °C), supernatant was transferred to further microcentrifuge tubes, then 300 µl distilled water and 300 µl of chloroform were added. Samples were vortexed and incubated at room temperature (25 °C) for 30 minutes.

Following centrifugation (13,150 RPM; 2 minutes; 25 °C), the lower lipid-containing layer was transferred into separate 6.0 ml transmethylation vials. Solvents in each vial were evaporated under a stream of nitrogen, then 2.0 ml transmethylating reagent (94.0 ml methanol, 6.0 ml sulfuric acid) and a few crystals of hydroquinone were

added. Vials were incubated for 5 hours at 70 °C, 1.0 ml distilled water and 1.5 ml hexane added, then shaken and allowed to stand for 10 minutes to form two separate layers. The upper lipid layers (hexane extract) containing the fatty acid- methyl esters (FAMEs) were transferred into small screw cap vials (1.0 ml capacity), then blown dry with nitrogen. Extracted FAMEs were dissolved in 20 µl carbon disulphide and 0.5 µl of the solution injected into the GLC apparatus, a Hewlett Packard 5890 series II gas liquid chromatograph equipped with a flame ionization detector. The column used was a 30 m Supelcowax 10 / 0.53 mm (Supelco, Supelco Park, Bellefonte, PA, USA). The inert carrier gas was helium with a flow rate of 3.27 ml/min. The oven, injector and detector temperatures were set at 180, 225 and 225 °C, respectively. Commercial standards of FAMEs, obtained from Supelco and Sigma Chemical Co. (St. Louis, MO, USA), were run under identical conditions and the chromatograms evaluated with reference to the retention times of the standards.

7.3.3.2. Analysis of phospholipid fatty acid composition

The procedure used for the extraction, transmethylation and subsequent analysis of phospholipids was the same as that used for total lipids, except that thin- layer chromatography (TLC) was first used to separate phospholipids from the 100 mg wet weight infective juvenile samples.

Following freeze-drying and chloroform: methanol extraction, the lipid extracts were transferred into screw cap vials (ca. 1 ml), blown dry under nitrogen, then re-

dissolved in 50 µl chloroform : methanol (2 : 1 v / v). Each sample (25 µl) was applied on silica-gel plates, the sample spots allowed to dry at room temperature (25 °C) for 1-2 minutes. then plates were transferred into the developing tank containing the solvent mixture (mobile phase) hexane/ diethyl ether/ acetic acid (85: 15: 2 v/ v/ v). The TLC plates were developed for 30 minutes then dried at room temperature (25 °C) for 1-2 minutes.

Phospholipid spots were visualized by placing the TLC plates for a few seconds in a separate developing tank containing a few crystals of iodine. The phospholipid spots outlined on the TLC plates were scraped off into separate transmethylation vials.

7.3.4. Statistical analysis

All fatty acids were expressed as mole % of the total lipid and phospholipid fractions. Unsaturation indices, which are a measure of the degree of unsaturation in terms of the number of double bonds/mole, were computed (Sumner and Morgan, 1969). Mole percentage data (arcsine transformed) and unsaturation indices were analysed using one-way ANOVA, Student- Newman- Keuls test (Jandel Corp$^{(R)}$. Sigma Stat, 1992). The level of significance was defined as $P< 0.05$.

7.4. RESULTS

The recycling or storage temperature regimes influenced the fatty acid composition of lipids in all the entomopathogenic nematodes (Tables 1-8). In both total lipids and

phospholipids. there was a general decline in the content of saturated fatty acids and in some isolates. monounsaturated fatty acids, together with an increase in polyunsaturated fatty acids at reduced temperatures. The unsaturation indices of the four isolates were the same at comparable temperatures. but in each isolate the indices increased with decrease in recycling or storage temperature (Tables 1-8).

The increased unsaturation of fatty acids at low temperatures in total lipids or phospholipids was at the expense of palmitic (16:0) and/or stearic (18:0) acids, the two most abundant fatty acids (Tables 1-8). The effect of decreased recycling temperature on the proportion of monounsaturated fatty acids in lipid moieties was less consistent. At the 5 °C storage temperature, there was a marked decrease in the percentage of monosaturated fatty acid(s) in the phospholipids of all isolates except *S. feltiae* NF strain, this being the only isolate that displayed a reduction of monounsaturated fatty acids in the total lipid fraction (Table 1). Such decreases in monounsaturated fatty acids appeared to be attributable to a significant decrease in oleic acid (18:1) in the phospholipid fractions of three of the four isolates and to decreases in palmitoleic (16:1) and eicosenoic (20:1) acids in the total lipids of *S. feltiae* NF strain (Table 1).

The increase in polyunsaturated fatty acids at reduced temperatures was attributable to significantly greater percentages of linoleic acid (18:2) in total lipids and phospholipids of all isolates (Tables 1-8). In all except *S. riobravis*, this was augmented by significantly increased proportions of eicosapenic acid (20:5*w*3) at 5 °C (Tables 4, 8). In *S. riobravis* stored at 5 °C, arachidonic acid (20:4), rather than eicosapenic acid, was elevated in the

phospholipid moiety. In *S. feltiae* Umeå strain. arachidonic acid (C20:4) actually decreased as a proportion of the phospholipids at temperatures ≤ 15 °C (Table 7).

In the two strains of *S. feltiae*. the temperatures below which significant increases in polyunsaturated fatty acids (Tables 1. 3. 5. 7) occurred were 15 °C for phospholipids (increased 18:2) and 10 °C for total lipids (increased 18:2 and 20:5). At temperatures ≤ 20 °C. *S. carpocapsae* All strain had increased polyunsaturated fatty acids in both phospholipid (increased 18:2) and total lipid (increased 18:2 and 20:5) fractions (Tables 2. 6). *Steinernema riobravis* TX strain showed significantly increased proportions of polyunsaturated fatty acids in total lipids at temperatures ≤ 20 °C (Table 4). Such increases were not significant in the phospholipid moiety (Table 8), however, until a recycling temperature of 10 °C had been reached (increased 18:2 and 20:5).

7.5. DISCUSSION

This study has shown that the composition of saturated and unsaturated fatty acids in the lipids of entomopathogenic nematodes was influenced by recycling and storage temperatures. Recycling at warmer temperatures increased the proportions of saturated fatty acids relative to unsaturated fatty acids in all four isolates of *Steinernema*. Conversely, when nematodes were recycled or stored at colder temperatures, the proportion of unsaturated fatty acids in their lipids were increased, resulting in increases in unsaturation indices.

A detailed analysis of the data revealed that the increased production of

polyunsaturated fatty acids at low temperatures was due to linoleic acid (18:2) and eicosapenic acid (20:5*w*3) or, in *S. riobravis* phospholipids (5 °C), arachidonic acid (20:4). In a related study, embodying only two warmer recycling temperatures (18 and 25 °C), Fodor *et al.* (1994) also reported that nematodes recycled for an unspecified time at 18 °C contained a higher proportion of eicosapenic acid (20:5*w*3) in their phospholipids. In other respects, particularly with regard to the proportions of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2), the present data are closer to those reported by Selvan *et al.* (1993a) for several steinernematids rather than the generally lower values reported by Fodor *et al.* (1994) for *S. carpocapsae* Mexican strain.

Adaptation to environmental temperatures by shifting the proportions of saturated: unsaturated fatty acids is a widespread phenomenon in poikilotherms. High levels of unsaturated fatty acids in phospholipids increase the membrane fluidity to maintain normal cellular functions at low environmental temperatures (Hazel, 1995; Hazel and Williams, 1990; Hazel and Prosser, 1974). The cyst stages of the plant parasitic nematode, *G. rostochiensis* were found to contain high levels of polyunsaturated fatty acids in their lipids, principally triacylglycerols and phospholipids, as an overwintering adaptation (Gibson *et al.*, 1995). Among insect parasitic nematodes, the proportion of unsaturated fatty acids in the phospholipids of *S. carpocapsae* was greater when nematodes were reared at 18 °C than when reared at 25 °C and this was accompanied by a less ordered arrangement of phospholipids within the cell membranes at the lower temperatures (Fodor *et al.*, 1994). Thus, in the present study, the changes in unsaturation of phospholipids in

whole nematode extracts resulting from different rearing or storage temperatures are probably adaptive in helping preserve membrane integrity over a wide range of temperatures. from 25 to 5 °C.

In all four nematode isolates. there was a temperature- induced shift in the unsaturation indices of total lipids that parallelled those of the phospholipids. It was determined via TLC that in addition to phospholipids, these nematodes contain substantial amounts of free fatty acids and triacylglycerols. with lesser quantities of diacylglycerols and monoacylglycerols (Jagdale and Gordon, unpublished research). This is because the infective juveniles of entomopathogenic nematodes contain similar high levels of lipids to those found in free-living nematodes and utilize them as an energy substrate (Selvan *et al.*, 1993a). Gordon *et al.* (1979) reported that the unsaturation index of stored triacylglycerols was greater in a boreally adapted mermithid nematode, *N. flumenalis*, than in the tropical mermithid, *R. culicivorax*, and suggested that such differences in unsaturation were necessary to maintain fluidity within the nematode's storage organ and permit accessibility of enzymes to lipid energy reserves at the temperatures prevailing within the respective habitats of the nematodes. It is possible that the four isolates of *Steinernema* in the present study adjusted the physical state of their storage nutriment to permit energy metabolism to occur over a broad range of environmental temperatures. A shift toward unsaturation would be adaptive in permitting enzyme accessibility to storage lipid at cold temperatures. However, nematodes held at a constant warm temperature (25 °C) were also found to increase their total lipid unsaturation as the storage time increased;

in this instance, time-related production of unsaturated fatty acids was regarded as detrimental, because unsaturated fatty acids contain a lower potential energy than saturated ones (Selvan *et al.*, 1993b).

The four nematode isolates examined in this study originated from habitats with diverse climates. The two strains of *S. feltiae* are boreal, *S. riobravis* is subtropical, while the All strain of *S. carpocapsae*, extensively subcultured, has a warm temperate origin (Poinar, 1979). Despite such differences in origin, these nematodes possessed similar degrees of unsaturation in their total lipids and phospholipids. These findings appear at variance with those of Selvan *et al.* (1993a), who reported that a tropical species, *Steinernema scapterisci*, contained a higher proportion of saturated fatty acids within its total lipids than several other entomopathogenic nematodes reared at the same temperature. Their data do not seem to support such a conclusion, however, as the proportion of saturated fatty acids in *S. scapterisci* was only marginally higher than some of the other species and a temperate strain of *S. feltiae* contained the highest proportion of polyunsaturated fatty acids. According to the present study, the temperatures below which significant elevations in polyunsaturated fatty acids occurred may be species-, but probably not habitat-, related. Thus, the temperature at or below which synthesis of polyunsaturated fatty acids is induced is higher in the total lipids (20 cf. 10 °C), but lower in the phospholipid fraction (10 cf. 15 °C) of the subtropical *S. riobravis*, compared to the two boreal strains of *S. feltiae*.

Many of the shifts in lipid unsaturation reported in this study were purely

environmentally induced. This applies to 5 °C determinations in all isolates, since no recycling was carried out at this temperature. Also, in *S. carpocapsae* at 10 °C and *S. riobravis* at 10 and 15°C, recycling was not possible. At other temperatures, however, it is possible that some artificial selection may have occurred, resulting in genetically altered synthetic capacities for fatty acids adaptive to the rearing temperatures. Given the above mentioned similarities in unsaturation indices among the four isolates, however, such a genetic influence seems unlikely, although it cannot be entirely discounted.

It has been observed that the upper and lower thermal tolerances of these entomopathogenic nematodes were influenced by the temperature at which they were recycled or stored. Recycling at warmer temperatures increased the upper lethal temperatures and decreased survival time in the frozen condition. Conversely, when nematodes were recycled at colder temperatures, their upper lethal temperatures were decreased, while their freezing survival times were lengthened (see Chapter 3). It was observed that the array of physiological mechanisms responsible for such temperature-induced shifts in thermal tolerances includes modifications in the capacities to synthesize isozymes of certain metabolic enzymes (see Chapter 5). From the present study, it would appear that shifts in the degree of unsaturation of lipids are also critical in enabling these nematodes to survive and reproduce at varying environmental temperatures. However, since the capacity for altering the unsaturation index appeared to be the same for all the isolates, differences in thermal tolerances among species are not explicable from the standpoint of lipid unsaturation.

These findings, that the lipid composition and the thermal tolerances of these nematodes changed adaptively with respect to recycling and storage temperatures suggest a degree of physiological plasticity that could be useful with respect to their commercialization. Thus, for a given species, rearing protocols can be designed to embody different temperatures in order to generate nematodes with the thermal tolerances and associated physiology appropriate to the biotopes into which they would be introduced.

Table 1. Effect of maintenance and storage temperature on the composition of fatty acids in total lipid of NF strain of *S. feltiae*.

Fatty acids	Maintenance / Storage Temperature °C				
	25	20	15	10	5
14:0	1.0 ± 0.4[†]	3.2 ± 1.0	1.3 ± 0.4	2.0 ± 0.3	1.2 ± 0.2
14:1	1.7 ± 0.6	7.5 ± 6.2	4.5 ± 1.3	0.2 ± 0.2	-
16:0	23.8 ±1.1[a]	26.7 ± 3.1[a]	22.2 ± 1.8[ab]	20.0 ± 2.1[ab]	15.6 ± 0.3
16:1*w*7	5.2 ± 1.4[a]	6.8 ± 1.9[a]	1.0 ± 0.5[b]	3.1 ± 0.9[ab]	0.9 ± 0.1[b]
18:0	23.7 ±2.7[a]	11.4 ± 3.4[b]	22.9 ± 0.5[a]	11.9 ± 1.2[b]	12.5 ± 0.4
18:1*w*9	22.2 ±0.8[a]	22.6 ± 5.2[a]	21.1 ± 0.5[a]	18.9 ± 2.7[a]	20.4 ± 1
18:2*w*6	16.0 ±2.7[a]	17.2 ± 3.4[a]	16.2 ± 0.7[a]	29.0 ± 1.4[b]	28.1 ± (
18:3*w*6	-	-	0.1 ± 0.1	0.5 ± 0.0	0.4 ± 0.2
18:3*w*3	-	-	0.5 ± 0.3	-	-
18:4*w*3	-	-	-	-	0.1 ± 0.1
20:0	-	-	0.4 ± 0.2	0.4 ± 0.3	0.5 ± 0.1
20:1*w*9	5.2 ± 2.3[a]	1.0 ± 1.0[b]	3.9 ± 0.7[a]	-	-
20:3*w*6	-	-	-	-	-
20:4*w*3	-	-	-	2.3 ± 0.1	4.4 ± 0.3
20:4*w*6	-	-	3.0 ± 1.3	-	-
20:5*w*3	2.6 ± 0.7[a]	3.7 ± 1.8[a]	4.0 ± 1.7[a]	12.6 ± 1.9[b]	15.5 ± 0.6
22:0	-	-	-	0.5 ± 0.3	0.7 ± 0.2
22:5*w*3	-	0.3 ± 0.3	0.4 ± 0.3	-	-
22:6*w*3	-	0.3 ± 0.0	0.3 ± 0.2	-	-
SFA[1]	48.5	41.3	46.8	34.8	30.5
MUFA[1]	34.5	37.9	30.5	22.2	21.3
PUFA[1]	18.6	21.5	24.5	44.4	48.5
UI[1]	0.8 ± 0.1[a]	0.9 ± 0.1[a]	1.1 ± 0.1[a]	1.5 ± 0.1[b]	1.7 ± 0.0[b]

[†]Values are expressed as Mole percentage and are the means ± SE of three replicates. Means with the same lower case letter (across the columns) are not significantly different (P > 0.05) by Student Newman-Keuls test.
[1]SFA= Saturated fatty acids, MUFA= Monounsaturated fatty acids, PUFA= Polyunsaturated fatty acids. UI= Unsaturation index

Table 2. Effect of maintenance and storage temperatures on the composition of fatty acids total lipids of All strain of *S. carpocapsae*.

Fatty acids	Maintenance / Storage Temperature °C				
	25	20	15	10	5
14:0	-	1.0 ± 0.1[†]	1.1 ± 0.1	1.0 ± 0.2	0.8 ± 0.1
14:1	-	-	-	-	-
16:0	22.0 ± 3.0[a]	14.5 ± 0.5[b]	13.6 ± 0.7[b]	12.1 ± 1.3[b]	13.4 ± 1.3[b]
16:1ω7	-	1.0 ± 0.1	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
18:0	26.4 ± 2.7[a]	18.8 ± 1.2[b]	18.7 ± 0.5[b]	17.3 ± 1.7[b]	11.2 ± 0.3[c]
18:1ω9	24.8 ± 0.6[a]	22.7 ± 0.4[a]	19.7 ± 1.1[b]	17.8 ± 0.4[b]	26.8 ± 0.4[a]
18:2ω6	18.3 ± 1.6[a]	24.7 ± 0.4[b]	25.7 ± 0.2[b]	30.4 ± 1.5[c]	29.2 ± 0.6[c]
18:3ω6	0.5 ± 0.5	0.3 ± 0.2	0.3 ± 0.1	0.5 ± 0.0	0.7 ± 0.0
18:3ω3	-	-	-	-	-
18:4ω3	-	0.3 ± 0.2	0.2 ± 0.1	-	0.1 ± 0.1
20:0	0.3 ± 0.3	1.0 ± 0.1	0.6 ± 0.2	1.5 ± 0.5	0.4 ± 0.1
20:1ω9	-	-	-	-	-
20:3ω6	-	-	-	-	-
20:4ω3	0.8 ± 0.8[a]	2.4 ± 0.2[b]	3.0 ± 0.2[b]	3.7 ± 0.4[b]	0.9 ± 0.2[b]
20:4ω6	-	-	-	-	-
20:5ω3	6.8 ± 1.1[a]	12.2 ± 1.0[b]	15.7 ± 0.4[b]	12.7 ± 0.6[b]	13.0 ± 1.1[b]
22:0	-	1.2 ± 0.1	0.9 ± 1.0	2.2 ± 0.6	0.7 ± 0.2
22:5ω3	-	-	-	-	-
24:0	-	-	-	-	-
22:6ω3	-	-		-	0.2 ± 0.2
SFA[1]	48.7	36.5	34.9	34.1	26.5
MUFA[1]	24.8	23.7	20.2	18.4	27.5
PUFA[1]	26.4	39.9	44.9	47.3	44.1
UI[1]	1.0 ± 0.1[a]	1.4 ± 0.1[b]	1.6 ± 0.0[b]	1.6 ± 0.0[b]	1.7 ± 0.0[b]

[†]Values are expressed as Mole percentage and are the means ± SE of three replicates. Mea with the same lower case letter (across the columns) are not significantly different (P > 0. by Student Newman-Keuls test.

[1]SFA= Saturated fatty acids, MUFA= Monounsaturated fatty acids, PUFA= Polyunsaturat fatty acids. UI= Unsaturation index

Table 3. Effect of maintenance and storage temperatures on the composition of fatty acids total lipids of Umeå strain of *S. feltiae*.

Fatty acids	Maintenance / Storage Temperature °C				
	25	20	15	10	5
14:0	2.7 ± 1.4[†]	2.6 ± 0.4	3.4 ± 0.3	1.2 ± 0.1	1.6 ± 0.2
14:1	1.8 ± 0.7	8.8 ± 1.0	6.7 ± 0.4	-	0.6 ± 0.1
16:0	28.8 ± 0.2[a]	20.7 ± 0.1[b]	26.4 ± 0.6[c]	15.2 ± 0.6[d]	15.1 ± 0.5[d]
16:1*w*7	0.4 ± 0.4[a]	8.1 ± 1.3[b]	8.5 ± 0.3[b]	0.8 ± 0.0[a]	3.8 ± 0.1[c]
18:0	19.4 ± 4.4[a]	4.2 ± 0.3[b]	5.2 ± 0.3[b]	14.9 ± 0.6[ac]	10.6 ± 0.7[c]
18:1*w*9	25.5 ± 1.5[a]	33.2 ± 1.0[b]	32.8 ± 1.0[b]	22.5 ± 0.2[c]	20.3 ± 0.4[c]
18:2*w*6	17.2 ± 1.4[a]	17.7 ± 1.4[a]	15.4 ± 0.1[a]	25.1 ± 0.2[b]	27.4 ± 0.8[b]
18:3*w*6	-	1.3 ± 0.7	1.2 ± 0.2	0.3 ± 0.1	0.6 ± 0.1
18:3*w*3	0.2 ± 0.2	0.6 ± 0.3	0.5 ± 0.3	-	0.1 ± 0.1
18:4*w*3	-	-	-	-	0.3 ± 0.1
20:0	0.1 ± 0.1	-	-	0.8 ± 0.1	1.0 ± 0.1
20:1*w*9	2.6 ± 1.4	1.7 ± 0.2	1.6 ± 0.2	-	-
20:3*w*6	-	0.6 ± 0.1	0.5 ± 0.1	-	-
20:4*w*3	-	-	-	4.3 ± 0.2	3.8 ± 0.1
20:4*w*6	1.2 ± 0.6	0.4 ± 0.1	0.3 ± 0.1	-	-
20:5*w*3	2.4 ± 1.2[a]	2.4 ± 0.3[a]	1.5 ± 0.1[a]	14.3 ± 0.4[b]	15.2 ± 0.9[b]
22:0	-	0.4 ± 0.2	0.1 ± 0.1	0.8 ± 0.2	1.0 ± 0.1
22:5*w*3	-	-	-	-	-
24:0	-	-	-	-	-
22:6*w*3	-	-	-	-	-
SFA[1]	51.0	27.9	35.1	32.9	29.3
MUFA[1]	30.3	51.8	49.6	23.3	24.7
PUFA[1]	21.0	23.0	19.4	44.0	47.4
UI[1]	0.8 ± 0.1[a]	1.1 ± 0.0[b]	1.0 ± 0.0[ab]	1.6 ± 0.0[c]	1.7 ± 0.1[c]

[†]Values are expressed as Mole percentage and are the means ± SE of three replicates. Mea with the same lower case letter (across the columns) are not significantly different (P > 0. by Student Newman-Keuls test.

[1]SFA= Saturated fatty acids, MUFA= Monounsaturated fatty acids, PUFA= Polyunsatura fatty acids. UI= Unsaturation index

Table 4. Effect of maintenance and storage temperatures on the composition of fatty acids total lipid of TX strain of *S. riobravis*.

Fatty acids	Maintenance / Storage Temperature °C				
	25	20	15	10	5
14:0	0.7 ± 0.4[†]	1.0 ± 0.1	1.5 ± 0.2	0.3 ± 0.3	1.2 ± 0.3
14:1	0.6 ± 0.5	-	-	-	-
16:0	21.4 ± 2.4[a]	24.0 ± 1.3[a]	22.3 ± 1.0[a]	17.9 ± 0.7[a]	17.3 ± 1.9[a]
16:1ω7	0.8 ± 0.4	0.9 ± 0.4	0.9 ± 0.1	0.4 ± 0.3	0.7 ± 0.1
18:0	24.5 ± 2.0[a]	14.8 ± 2.0[b]	14.3 ± 1.5[b]	19.1 ± 1.4[ab]	12.4 ± 1.3[bc]
18:1ω9	29.4 ± 1.1[a]	31.2 ± 1.0[a]	32.1 ± 1.3[a]	29.3 ± 0.4[a]	25.8 ± 3.3[a]
18:2ω6	15.3 ± 0.8[a]	19.2 ± 1.0[b]	19.5 ± 0.9[b]	17.3 ± 0.6[ab]	27.5 ± 0.6[c]
18:3ω6	-	0.6 ± 0.0	0.6 ± 0.0	1.1 ± 1.1	0.7 ± 0.1
18:3ω3	0.1 ± 0.1	-	-	-	-
18:4ω3	-	-	-	-	0.1 ± 0.1
20:0	0.3 ± 0.2	1.0 ± 0.4	0.7 ± 0.1	0.2 ± 0.2	0.9 ± 0.2
20:1ω9	0.4 ± 0.2	-	-	-	-
20:3ω6	-	-	-	-	-
20:4ω3	1.7 ± 0.9[a]	3.8 ± 0.3[ab]	4.0 ± 0.3[ab]	6.5 ± 0.4[b]	5.6 ± 1.0[b]
20:4ω6	0.1 ± 0.1	-	-	-	-
20:5ω3	5.5 ± 1.5[a]	4.7 ± 0.4[a]	5.4 ± 0.4[a]	8.0 ± 0.6[a]	8.0 ± 1.1[a]
22:0	0.3 ± 0.2	0.4 ± 0.2	0.4 ± 0.0	0.6 ± 0.6	0.5 ± 0.8
22:5ω3	-	-	-	-	-
24:0	-	-	-	-	-
22:6ω3	0.1 ± 0.9				
SFA[1]	47.2	41.2	39.2	38.1	32.3
MUFA[1]	31.7	32.1	33.0	29.7	26.5
PUFA[1]	22.8	28.3	29.5	32.9	41.9
UI[1]	1.0 ± 0.1[a]	1.1 ± 0.0[a]	1.2 ± 0.0[ab]	1.3 ± 0.0[bc]	1.5 ± 0.1[c]

[†]Values are expressed as Mole percentage and are the means ± SE of three replicates. Mea with the same lower case letter (across the columns) are not significantly different (P > 0.0 by Student Newman-Keuls test.

[1]SFA= Saturated fatty acids, MUFA= Monounsaturated fatty acids, PUFA= Polyunsaturate fatty acids. UI= Unsaturation index

Table 5. Effect of maintenance and storage temperatures on the composition of fatty acids phospholipids of NF strain of *S. feltiae*.

Fatty acids	Maintenance / Storage Temperature °C				
	25	20	15	10	5
14:0	1.0 ± 0.2[†]	1.1 ± 0.6	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.2
14:1	-	-	-	-	-
16:0	15.1 ± 1.2[a]	16.0 ± 1.0[a]	14.5 ± 0.3[a]	13.3 ± 0.5[ab]	11.4 ± 1.2[b]
16:1ω7	0.1 ± 0.0	1.2 ± 1.0	1.1 ± 0.0	0.5 ± 0.1	0.4 ± 0.2
18:0	21.7 ± 3.7[a]	18.0 ± 2.1[b]	16.2 ± 1.0[b]	15.7 ± 1.6[b]	12.0 ± 0.7[c]
18:1ω9	14.0 ± 0.2[a]	11.0 ± 0.7[b]	13.5 ± 0.1[a]	12.6 ± 0.3[a]	14.6 ± 0.1[a]
18:2ω6	24.6 ± 2.4[a]	27.8 ± 1.0[ac]	34.1 ± 0.2[b]	31.5 ± 1.0[bc]	31.8 ± 0.5[bc]
18:3ω6	0.4 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.5 ± 0.1	0.6 ± 0.0
18:3ω3	-	-	-	-	-
18:4ω3	0.1 ± 0.1	3.0 ± 3.0	-	0.2 ± 0.1	0.1 ± 0.1
20:0	0.5 ± 0.3	0.5 ± 0.2	0.3 ± 0.1	0.5 ± 0.0	0.6 ± 0.1
20:4ω3	5.6 ± 0.4[a]	4.0 ± 0.3[b]	5.0 ± 0.2[a]	5.0 ± 0.1[a]	5.8 ± 0.2[a]
20:5ω3	13.6 ± 1.6[a]	16.1 ± 1.0[ab]	14.3 ± 1.0[ab]	18.2 ± 0.4[b]	20.4 ± 0.8[bc]
22:0	0.6 ± 0.1	0.4 ± 0.2	-	0.4 ± 0.0	0.7 ± 0.1
22:5ω3	-	-	-	-	-
24:0	0.6 ± 0.2	0.5 ± 0.2	-	0.1 ± 0.1	-
22:6ω3	0.5 ± 0.1	0.5 ± 0.3	-	0.3 ± 0.0	0.8 ± 0.2
SFA[1]	39.5	36.5	32.0	31.0	25.7
MUFA[1]	14.1	12.2	14.6	13.2	15.0
PUFA[1]	44.8	51.5	53.6	55.7	59.5
UI[1]	1.6 ± 0.1[a]	1.8 ± 0.1[ab]	1.8 ± 0.0[ab]	1.9 ± 0.0[ab]	2.1 ± 0.1[b]

[†]Values are expressed as Mole percentage and are the means ± SE of three replicates. Mea with the same lower case letter (across the columns) are not significantly different (P > 0. by Student Newman-Keuls test. [1]SFA= Saturated fatty acids, MUFA= Monounsaturat fatty acids, PUFA= Polyunsaturated fatty acids. UI= Unsaturation index

Table 6. Effect of maintenance and storage temperatures on the compostion of fatty acids phospholipids of All strain of *S. carpocapsae*.

Fatty acids	Maintenance / Storage Temperature °C				
	25	20	15	10	5
14:0	0.7 ± 0.4[†]	-	0.4 ± 0.4	-	0.6 ± 0.1
14:1	-	-	-	-	-
16:0	14.9 ± 0.1[a]	12.6 ± 2.2[a]	13.9 ± 1.7[a]	16.0 ± 2.1[a]	10.5 ± 0.9[a]
16:1*w*7	0.4 ± 0.4	-	1.9 ± 0.4	-	0.6 ± 0.2
18:0	24.0 ± 2.5[a]	22.5 ± 1.9[a]	19.2 ± 1.1[ab]	20.7 ± 1.2[ab]	15.4 ± 0.4[b]
18:1*w*9	24.4 ± 0.4[a]	19.7 ± 0.6[b]	20.1 ± 0.6[b]	16.1 ± 0.7[c]	12.8 ± 0.4[d]
18:2*w*6	21.0 ± 1.0[a]	26.4 ± 0.8[b]	31.6 ± 0.4[c]	33.2 ± 1.1[c]	37.2 ± 0.3[d]
18:3*w*6	0.1 ± 0.1	-	-	-	0.3 ± 0.1
18:3*w*3	-	-	-	-	-
18:4*w*3	-	-	-	-	-
20:0	-	0.9 ± 0.7	-	-	0.3 ± 0.1
20:4*w*3	3.0 ± 0.1[a]	3.5 ± 0.2[a]	2.8 ± 0.5[a]	3.2 ± 0.2[a]	4.0 ± 0.2[a]
20:5*w*3	10.4 ± 0.7[a]	13.3 ± 1.0[ab]	10.4 ± 1.1[a]	10.1 ± 1.0[a]	16.0 ± 0.7[b]
22:0	1.1 ± 0.4	0.2 ± 0.2	-	-	0.7 ± 0.2
22:5*w*3	-	-	-	-	-
24:0	-	0.5 ± 0.5	-	0.4 ± 0.4	0.2 ± 0.2
22:6*w*3	0.3 ± 0.3	-	0.4 ± 0.4	-	1.0 ± 0.3
SFA[1]	40.7	36.5	33.5	37.1	27.7
MUFA[1]	24.8	19.7	22.0	16.1	13.4
PUFA[1]	34.8	43.2	45.2	46.5	58.5
UI[1]	1.3 ± 0.1[a]	1.5 ± 0.1[a]	1.5 ± 0.1[a]	1.5 ± 0.1[a]	1.9 ± 0.0[b]

[†]Values are expressed as Mole percentage and are the means ± SE of three replicates. Mea with the same lower case letter (across the columns) are not significantly different (P > 0. by Student Newman-Keuls test. [1]SFA= Saturated fatty acids MUFA= Monounsaturated fa acids, PUFA= Polyunsaturated fatty acids. UI= Unsaturation index

Table 7. Effect of maintenance and storage temperatures on the composition of fatty ac phospholipids from Umeå strain of *S. feltiae*.

Fatty acids	Maintenance / Storage Temperature °C				
	25	20	15	10	5
14:0	1.1 ± 0.6[†]	1.2 ± 0.0	1.5 ± 0.2	1.7 ± 0.0	1.2 ± 0.0
14:1	-	-	-	-	-
16:0	17.6 ± 1.8[a]	16.8 ± 0.3[a]	17.3 ± 0.5[a]	15.9 ± 0.6[a]	13.9 ± 0.5[a]
16:1*w*7	0.7 ± 0.3[a]	1.0 ± 0.0[ac]	0.1 ± 0.2[b]	0.9 ± 0.2[c]	0.9 ± 0.1[c]
18:0	19.8 ± 0.5[a]	19.0 ± 1.0[a]	12.2 ± 0.6[b]	11.5 ± 0.2[b]	13.2 ± 1.3[b]
18:1*w*9	16.9 ± 0.3[a]	18.4 ± 1.0[a]	12.9 ± 0.3[b]	11.9 ± 0.2[b]	9.5 ± 0.1[c]
18:2*w*6	23.1 ± 0.5[a]	22.4 ± 0.7[a]	33.2 ± 0.2[b]	33.5 ± 0.4[b]	35.5 ± 0.4[b]
18:3*w*6	0.1 ± 0.1	-	0.6 ± 0.1	0.5 ± 0.2	0.4 ± 0.0
18:3*w*3	-	-	0.3 ± 0.3	-	-
18:4*w*3	0.2 ± 0.1	0.1 ± 0.1	0.6 ± 0.3	0.2 ± 0.2	0.5 ± 0.3
20:0	0.4 ± 0.2	0.6 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.1
20:4*w*3	4.0 ± 0.3[a]	4.7 ± 0.0[b]	3.6 ± 0.1[a]	2.9 ± 0.1[c]	2.9 ± 0.2[c]
20:5*w*3	14.4 ± 1.0[a]	15.7 ± 0.5[a]	14.4 ± 0.5[a]	19.2 ± 1.0[b]	20.4 ± 1.0[b]
22:0	1.6 ± 1.1	0.4 ± 0.2	0.2 ± 0.1	0.2 ± 0.2	0.6 ± 0.1
22:5*w*3	-	-	-	-	-
24:0	0.1 ± 0.1	-	0.5 ± 0.1	0.5 ± 0.3	0.4 ± 0.0
22:6*w*3	0.1 ± 0.1	-	0.2 ± 0.1	-	0.5 ± 0.1
SFA[1]	40.6	38.0	32.1	30.2	29.8
MUFA[1]	17.6	19.4	13.0	12.8	10.4
PUFA[1]	42.0	42.9	52.9	56.3	59.5
UI[1]	1.5 ± 0.1[a]	1.6 ± 0.0[ab]	1.7 ± 0.0[b]	1.9 ± 0.0[cd]	2.0 ± 0.0[d]

[†]Values are expressed as Mole percentage and are the means ± SE of three replicates. Me with the same lower case letter (across the columns) are not significantly different (P > 0 by Student Newman-Keuls test. [1]SFA= Saturated fatty acids, MUFA= Monounsaturated fa acids, PUFA= Polyunsaturated fatty acids. UI= Unsaturation index

Table 8. Effect of maintenance and storage temperatures on the composition of fatty ac phospholipid of TX strain of *S. riobravis*.

Fatty acida	Maintenance / Storage Temperature °C				
	25	20	15	10	5
14:0	1.0 ± 0.1[†]	0.7 ± 0.0	0.5 ± 0.2	0.6 ± 0.1	1.0 ± 0.1
14:1	-	-	-	-	-
16:0	17.0 ± 0.4[a]	13.2 ± 0.7[b]	16.2 ± 1.2[a]	13.0 ± 0.6[b]	14.8 ± 0.7[c]
16:1*w*7	0.4 ± 0.0	0.4 ± 0.0	0.7 ± 0.3	0.6 ± 0.0	0.4 ± 0.0
18:0	15.7 ± 0.4[a]	19.7 ± 2.2[a]	15.4 ± 0.6[a]	15.3 ± 0.5[a]	17.2 ± 1.2[a]
18:1*w*9	31.1 ± 0.4[a]	27.7 ± 2.1[ac]	18.9 ± 0.2[b]	26.7 ± 0.4[c]	17.2 ± 0.6[bd]
18:2*w*6	19.9 ± 0.2[a]	18.4 ± 2.4[a]	30.1 ± 0.9[b]	19.0 ± 0.3[a]	29.0 ± 1.0[b]
18:3*w*6	0.6 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	0.7 ± 0.2
18:3*w*3	-	-	-	-	-
18:4*w*3	-	-	0.1 ± 0.1	-	-
20:0	0.5 ± 0.0	1.0 ± 0.1	0.4 ± 0.2	0.7 ± 0.1	0.7 ± 0.0
20:4*w*3	6.3 ± 0.2[a]	5.8 ± 0.8[a]	8.0 ± 0.4[ab]	7.7 ± 0.3[ab]	8.5 ± 0.4[b]
20:5*w*3	7.2 ± 0.1[a]	10.0 ± 2.1[a]	8.6 ± 0.4[a]	13.6 ± 0.5[b]	9.4 ± 0.6[a]
22:0	0.1 ± 0.1	0.7 ± 0.3	0.2 ± 0.1	0.7 ± 0.1	0.3 ± 0.0
22:5*w*3	-	-	-	-	-
24:0	0.5 ± 0.1	0.5 ± 0.2	0.5 ± 0.4	0.7 ± 0.2	0.5 ± 0.2
22:6w3	0.4 ± 0.0	1.2 ± 0.6	0.3 ± 0.1	1.0 ± 0.1	0.5 ± 0.1
SFA[1]	34.8	35.8	33.2	30.9	34.4
MUFA[1]	31.5	28.1	19.5	27.3	17.6
PUFA[1]	34.4	35.8	47.5	41.3	48.1
UI[1]	1.4 ± 0.0[a]	1.5 ± 0.1[a]	1.6 ± 0.0[a]	1.7 ± 0.0[a]	1.6 ± 0.1[a]

[†]Values are expressed as Mole percentage and are the means ± SE of three replicates. Mea with the same lower case letter (across the columns) are not significantly different (P > 0. by Student Newman-Keuls test. [1]SFA= Saturated fatty acids, MUFA= Monounsaturat fatty acids, PUFA= Polyunsaturated fatty acids. UI= Unsaturation index

CHAPTER 8

GENERAL DISCUSSION

Since new isolates of entomopathogenic nematodes are being reported with increasing frequency, it is important to be able to identify them, so that a world wide inventory of available stocks can be compiled (Akhurst, 1995). Accurate identification of entomopathogenic nematodes is difficult using classical morphological methods, because like certain categories of plant parasitic nematodes (Kaplan, 1996; Nogueira, 1996), each species exists as a number of geographically distinct strains (Poinar, 1990). Moreover, no strain-specific DNA probes have been reported for entomopathogenic nematodes (Hominick and Reid, 1990).

Cellulose acetate electrophoresis proved effective in discriminating between five strains (All strain of *Steinernema carpocapsae*, L1C, NF and Umeå strains of *S. feltiae* and TX strain of *S. riobravis*) of entomopathogenic nematodes (Jagdale *et al.*, 1996). Based on RFLP's, the newly isolated NF strain of *S. feltiae* was indistinguishable from the L1C strain isolated 14 years ago in Newfoundland (Finney-Crawley, 1985), but the isozyme patterns of the enzyme arginine kinase, permitted separation of these closely-related strains.

Cellulose acetate electrophoresis also proved useful in enabling identification of a strain that was first isolated from soil in Sweden (Pye and Pye, 1985) and since then designated as the Umeå strain of *S. carpocapsae*. However, based on a combination of its restriction fragment length polymorphisms (RFLP's) and its morphometry, and isozymic patterns of six enzymes (Chapter 2), this strain is now considered to be a strain of *S. feltiae* (Jagdale *et al.*, 1996; West and Vrain, 1997) and could be distinguished from the

other two strains of *S. feltiae*, NF and L1C. DNA analysis alone was insufficient to distinguish between these strains (Jagdale *et al.*, 1996). These findings suggest that cellulose acetate could be an important taxonomic tool for identification of isolates of species of *Steinernema*. Its relatively low cost and its rapidity make it a particularly attractive procedure. Since the isozyme patterns are temperature-sensitive, changing according to recycling temperature (Chapter 5), the temperature at which the nematodes are recycled should be specified in order for the technique to be of taxonomic value.

Long-term (two years) recycling at various temperatures modified the capacities of the nematode strains for temperature tolerance and infectivity (Chapters 3 and 4), biological characteristics that are important to field efficacy. Recycling at warmer temperatures increased the upper lethal temperatures and decreased survival times in the frozen condition in all strains. Conversely, when nematodes were reared at colder temperatures, their upper lethal temperatures were decreased, while their freezing survival times were lengthened (Chapter 3). Recycling of these nematodes at warmer temperatures completely inhibited the capacity for infection at the lower limit of each strain's temperature range (for infection) and at least for the Umeå strain of *S. feltiae*, the capacity for infection at warmer temperatures (20 to 25 °C) was reduced by recycling at colder (≤ 15 °C) temperatures (Chapter 4). Griffin (1993) suggested that pest management programs would have the greatest chance of success if the original source of nematodes selected for use was from a habitat with a climate similar to the environment in which their use is contemplated. The present study suggests that the thermal history of the

candidate nematodes during industrial mass cultivation constitutes an additional factor to be considered in determining the most suitable isolate to be used, along with the ideal temperature conditions for its application.

In all four strains (All, NF, Umeå and TX), the changes in infectivity and thermal tolerance resulting from recycling at various temperatures were accompanied by physiological changes. As the recycling temperatures decreased, there was an increase in specific activities of two key metabolic enzymes (Chapter 6), a shift toward unsaturated (cf. saturated) fatty acids (Chapter 7) and in some instances, production of additional isozymes of metabolic enzymes (Chapter 5). It is tempting to conclude that the physiological changes recorded are at least part of the mechanism that underlies the shifts in biological characteristics.

The increased bias toward unsaturated fatty acids at low temperatures is consistent with results of studies on teleosts (Hazel, 1995; Hazel and Prosser, 1974; Hazel and Williams, 1990) At colder temperatures, increased unsaturation of fatty acids is adaptive, since, by lowering the lipid transition point, it enables maintenance of membrane integrity (Hazel, 1995; Hazel and Prosser, 1974; Hazel and Williams, 1990; Sinensky, 1974) and accessibility of enzymes to neutral lipid stores (Gordon *et al.*, 1979).

The elevated specific activities of metabolic enzymes, glucose-6-phosphate dehydrogenase and hexokinase, also appear to be adaptive to declining temperatures, since they provide a compensatory mechanism for what would otherwise constitute an exponential deceleration of metabolic pathways (Schmidt Nielsen, 1995). Such

compensatory effects on enzyme activities have been observed for teleosts (Campbell and Davies. 1978: Hochachka and Clayton-Hochachka. 1973: Hochachka and Hayes. 1962: Yamauchi *et al.*. 1975) and invertebrates (Joanisse and Storey, 1994), although they have not been thus far reported for nematodes. In studies done on teleosts, the cold temperature- induced increase in enzyme activity was correlated with a shift in enzyme-substrate affinity. such that the lowest K_m value for the substrate occurred at a temperature corresponding to the acclimation temperature (Baldwin and Hochachka. 1970). In such instances. the shift in enzyme activity can be attributed to a basic change in enzyme - substrate affinity, possibly involving a switch to production of a different isozyme (Baldwin and Hochachka, 1970; DiMichele *et al.*, 1991; Hazel and Prosser, 1974; Lin *et al.*. 1996). The steinernematids in the present study did not respond to declining temperatures by modulating their K_m values in so interpretive a fashion. In only three instances (*S. feltiae* NF strain and *S. carpocapsae* All strain: glucose-6-phosphate dehydrogenase; *S. riobravis* TX strain: hexokinase) did enzyme substrates display minimum K_m values that were correlated with the recycling temperatures. Thus, the adaptive changes in enzyme activity are explicable in terms other than changes in K_m values (e.g. enhanced enzyme synthesis). The enzymes of the nematodes responded to temperature in a similar fashion to citrate synthase in certain crustaceans by modifying the magnitude of K_m values, though not necessarily the temperature at which the values are minimal (Vetter, 1995).

The enzymes hexokinase and glucose-6-phosphate dehydrogenase mediate steps

in the first stages of glycolysis and the pentose phosphate pathway, respectively. Accentuated activities of these two enzymes would lead to increased production of pentoses and metabolic reducing equivalents (NADP). Promulgation of the pentose phosphate pathway has been shown also to occur in cold acclimated teleosts and it has been suggested that this may serve to increase lipid and nucleic acid biosynthesis (Hochachka and Somero. 1973). Whether this is indeed the end result in steinernematids requires investigation. Since it is generally thought that steinernematids rely on abundant reserves of neutral lipids for energy metabolism (Selvan *et al.*, 1993a), there would be adaptive value in channelling metabolites toward lipogenesis.

While the data on fatty acids and enzyme activities would appear to be in keeping with generally accepted concepts of thermal adaptation, the isozyme distribution patterns at the various recycling temperatures are less clearly correlated to the changes in biological characteristics of the nematodes. While it was the case that additional isozymes of certain metabolic enzymes (mannose 6-phosphate isomerase, malate dehydrogenase, phosphoglucomutase) were synthesized by nematodes recycled at cold or warm temperatures, there were several instances in which directional trends were not obvious. In such instances, it may be that inadvertent genetic selection, with no adaptive benefit, has occurred, resulting from isolation of the separately maintained colonies.

It is also possible that at least some of the modifications in the biological and physiological characteristics of the steinernematid nematodes resulting from long-term recycling at specified temperatures were due to changes in their genome, resulting from

artificial selection. This would apply to changes in whole organism characteristics and physiological parameters that occurred at temperatures at which the isolate was propagated. rather than simply acclimated (i.e. *S. feltiae* NF and Umeå strains at 10- 25 °C. *S. carpocapsae* All strain at 15- 25 °C and *S. riobravis* at 20- 25 °C). It was similarly concluded that the superior capacities for infection of *H. bacteriophora* HP88 strain and *S. anomali* Riazan strain (Grewal *et al.*, 1996) and for heat tolerance in *H. bacteriophora* IS5 strain (Shapiro *et al.*, 1996) that occurred at a 30 °C recycling temperature (compared to lower temperatures) were genetically based.

Environmentally induced changes in physiological characteristics, similar to what have been reported in teleosts (Hazel and Prosser, 1974), clearly occurred in all four isolates at temperatures where recycling was not possible and infective juveniles were stored without propagation. Temperature- specific additional isozymes of three metabolic enzymes were synthesized in *S. feltiae* NF and Umeå strains stored at 5 °C, *S. carpocapsae* All strain stored at 5 and 10 °C and *S. riobravis* TX strain stored at 10 and 15 °C (Chapter 5). Similarly, there was a shift toward unsaturation of fatty acids in nematodes that were stored but not recycled (*S. feltiae* NF and Umeå strains stored at 5 °C, *S. carpocapsae* All strain stored at 5 and 10 °C and *S. riobravis* TX strain stored at 10 and 15 °C). The proposition that physiological changes resulting from thermal acclimation bring about changes in biological characteristics was suggested by Griffin (1996), who found that the infectivity of *Heterorhabiditis* sp. at a cold (9 °C) temperature was enhanced by first storing the infective juveniles at the same temperature.

In the present study, it appears that regardless of original habitat or geographic location. all three species of *Steinernema* shared a common set of biological and physiological responses to recycling or storage at specified temperatures. In all four isolates. enzyme specific activities and proportions of unsaturated fatty acids in their lipids increased with decrease in temperature. Moreover, even the warm temperate *S. carpocapsae* All strain and the sub-tropical *S. riobravis* TX strain contained a preponderance of unsaturated fatty acids in their lipids and the unsaturation indices of all isolates was ≥ 1 regardless of recycling temperature. *Steinernema riobravis* TX strain did not synthesize cold- temperature specific isozymes like the NF and Umeå strains of *S. feltiae* and the All strain of *S. carpocapsae*. However, like the other species, it displayed a capacity for freezing-survival. These findings suggest that all three species of steinernematid nematodes, originally isolated from various geographic regions. have a common ancestory and that the nematodes show capacity to adapt to cold temperatures and freezing conditions. They give support to the suggestion that the common ancestor of this family of nematodes lived first in the temperate or boreal habitats, then dispersed globally into other climatic zones. *Steinernema carpocapsae* and *S. feltiae* appear to have a global distribution and the latter species is considered to be an ancient one which was present before continents began breaking and drifting apart (Hominick *et al.*, 1996).

The differences between the species regarding their thermal tolerance limits or infectivities seems to be correlated with the temperature profiles of their original habitat. Regardless of the temperature at which recycling was carried out, boreally adapted *S.*

feltiae is more cold tolerant and infective at colder temperatures than the warm temperate *S. carpocapsae* or sub-tropical *S. riobravis*. Conversely, regardless of recycling temperature, sub-tropical *S. riobravis* TX was the most heat tolerant and infective at high temperatures, while the warm temperate *S. carpocapsae* showed an intermediate degree of heat tolerance. From an evolutionary perspective, differences in biological characteristics (thermal tolerances or infectivities) of these three species of nematodes could be due to long-term changes in their allele frequencies resulting from the combined effects of genetic isolation and environmental stressors such as temperature. Such geographic changes in allele frequency within a population are known as clines (Richardson *et al.*, 1986).

The present study has shown that entomopathogenic nematodes can enhance their cold tolerance abilities by synthesizing new isozymes, modifying the specific activities of metabolic enzymes and increasing the degree of fatty acid unsaturation in their lipids. Such physiological adjustments would be adaptive in permitting nematode survival and activity at cold temperatures (5 °C) above freezing. However, the abilities of steinernematid nematodes to withstand freezing (Brown and Gaugler, 1996), as validated in the present study, undoubtedly involve deployment of other strategies. Recently it has been demonstrated *S. feltiae*, *S. anomali* and *H. bacteriophora* were freezing tolerant, withstanding extracellular ice formation (Brown and Gaugler, 1996) whereas *H. zealandica* was freeze avoiding, preventing freezing by supercooling its pseudocoelomic fluid to -32 °C (Wharton and Surrey, 1994). Further studies should be done to investigate

the potential roles of cryoprotectants (antifreeze proteins; glycerol; sorbitol), such as have been reported for several species of cold tolerant insects (Storey and Storey, 1992), in conferring protection against freezing.

In this study, wax moth (*G. mellonella*) larvae were used for rearing the nematodes *in vivo*. However, for commercial purposes in vitro methodologies are used (Friedman, 1990: Popiel and Hominick, 1992). Thus, it seems important to establish the degree to which the whole organism and physiological effects recorded in the present *in vivo* study occur in an *in vitro* commercial setting. The influence of the host in conferring biological and physiological properties to the nematodes is indicated by the findings of Surrey (1996), who demonstrated that infective juveniles of *H. zealandica* that had been propagated in *G. mellonella* larvae were more cold tolerant than those reared in artificial media.

From a commercial perspective, this study has shown that the cultivation protocol, especially rearing temperature, can modify biological characteristics of the nematode that influence field efficacy. While it would seem advantageous to select isolates based on the compatibility of the climate of their sites of origin with the test biotopes, the demonstrated ability to modify infectivity and thermal tolerances could allow greater versatility in the use of these nematodes and lead to enhanced field efficacy. By judicious manipulation of the recycling temperatures, it should be theoretically possible to generate several cultures of a given isolate, each with its own temperature preferences.

Continued studies are needed to more fully assess the impact of rearing protocols

for steinernematids on their pest management capacities. Such studies should be extended to include field trials. The importance of obtaining more complete physiological data on this highly promising family of insect pathogens is emphasized by the current findings.

9. REFERENCES

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Appendix I

Details of the Recycling Procedure

The method was based on Woodring and Kaya (1988). At each recycling temperature, for any given strain, 2000 infective juveniles were used to infect 100 wax moth (*G. mellonella)* larvae. Infections were arranged in Petri dishes (9 cm; 10 insects, 200 infective juveniles/dish). After the insects died (1-2 days), they were transferred and grouped together in White traps. Following emergence of the infective juveniles from the White traps, they were used to re-institute new infections.