Glycogen: its importance in the infectivity of aged juveniles of *Steinernema carpocapsae*

M. N. PATEL* *and* D. J. WRIGHT

Department of Biology, Imperial College of Science, Technology and Medicine, Silwood Park, Ascot, Berks SL5 7PY, UK

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SUMMARY

Infective juveniles (IJs) of *Steinernema carpocapsae* (All) are able to remain relatively highly infective even when they have almost exhausted their neutral lipid reserves. This is not seen in other steinernematid species so we proposed that carbohydrate may be important for infectivity in aging IJs of *S. carpocapsae*. The present study investigated glycogen utilization in IJs of 4 entomopathogenic nematodes, *S. carpocapsae*, *S. riobravis* (Biosys 355), *S. feltiae* (UK76) and *S. glaseri* (NC), stored in distilled water at 25 °C. The 4 species had appreciable amounts of glycogen; from *ca.* 8% dry weight in *S. riobravis* to *ca.* 18% in *S. glaseri*. Infective juveniles of *S. carpocapsae* and *S. riobravis* survived for 120–135 days and utilized *ca.* 90% of their glycogen reserve at an almost constant rate during a 112-day storage period. *Steinernema feltiae* and *S. glaseri* lived for much longer (> 450 days) and during a 250-day storage period their glycogen content decreased by 27 and 40%, respectively. In contrast to the other 3 species, the rate of lipid decline preceded that of glycogen in *S. carpocapsae*. The rate of glycogen decline in *S. carpocapsae* IJs incubated with the glycolytic inhibitor, iodoacetamide (10^{-4} M) was significantly reduced ($P < 0{\cdot}05$) compared with untreated nematodes, and the infectivity of inhibitor-treated aged (> 80 days) IJs was reduced compared with controls. Incubating aged (80-day) IJs of *S. carpocapsae* (mean neutral lipid content *ca.* 10% of initial level) with 10^{-4} M iodoacetamide for 24 h significantly reduced ($P < 0{\cdot}05$) their infectivity compared with freshly harvested inhibitor-treated IJs and untreated controls. Following an 11-day recovery period, the infectivity of inhibitor-treated aged IJs recovered significantly ($P < 0{\cdot}05$). The evidence suggests that glycogen is an important source of energy for maintaining infectivity in aged IJs of *S. carpocapsae*.

Key words: glycogen, utilization, infectivity, entomopathogenic nematodes, iodoacetamide.

INTRODUCTION

Carbohydrates are not considered to be a major energy reserve in free-living stages of animal-parasitic nematodes, and in many infective juveniles glycogen is difficult to detect (Barrett, 1976). In infective juveniles (IJs) of rhabditid entomopathogenic nematodes, lipids form the major energy reserve; total lipid constituting between 30 and 50% of the dry weight of the nematodes (Selvan, Gaugler & Lewis, 1993; Patel, Stolinski & Wright, 1997). However, glycogen is also reported to be present in appreciable amounts (as much as 10% dry weight; Selvan *et al.* 1993) although its role in the survival of entomopathogenic nematode IJs has yet to be determined. In a recent study, the infectivity of 3 entomopathogenic nematode species, *Steinernema riobravis* (Biosys 355), *S. feltiae* (UK76) and *S. glaseri* (NC) was shown to decline with neutral lipid utilization, but a fourth species, *S. carpocapsae* (All), was observed to maintain high levels of infectivity when neutral lipid levels were practically exhausted (Patel *et al.* 1997). It was therefore proposed that carbohydrates (glycogen) are an important source of energy in aged IJs of *S. carpocapsae* for the maintenance of infectivity. To investigate this further (a) glycogen utilization in *S. carpocapsae* IJs, and (b) the effect of a glycolytic inhibitor on the infectivity of aged, lipid-depleted *S. carpocapsae* was examined. Glycogen utilization in IJs of the other 3 *Steinernema* species was also quantified for comparative purposes.

* Corresponding author. Tel: 01344 294243/303/424. Fax: 01344 294339. E-mail: m.n.patel@bio.ic.ac.uk.

MATERIALS AND METHODS

Nematode cultures

Infective juveniles of *S. carpocapsae* (All), *S. riobravis* (Biosys 355), *S. feltiae* (UK76) and *S. glaseri* (NC) were cultured at 20 °C in late instar larvae of the wax moth, *Galleria mellonella* (Woodring & Kaya, 1988). Three hundred insect larvae were used to culture each nematode species and only IJs emerging within 4 days after first emergence were used in the study.

Glycogen utilization

Freshly harvested IJs were stored in aerated plastic culture boxes (15 × 11 × 6 cm; 60 boxes per species)

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in distilled water at 25 °C (200 ml per box at 2000 IJs/ml). The storage water was changed weekly and the incubation medium shaken daily. Three samples (> 100000 IJs per 20 boxes) were taken on days, 0, 21, 41, 84 and 112 for *S. carpocapsae* and *S. riobravis*, and on days 0, 41, 112, 178 and 250 for *S. feltiae* and *S. glaseri* during the storage period. The number of IJs in each sample and their dry weights were estimated before determination of glycogen content. Glycogen was extracted by alkaline digestion and determined using a modified Montgomery phenol–sulfuric acid assay (MacInnis, 1970). Lyophilized nematode tissue was hydrolysed with 30 % (w/v) KOH (2 ml) for 1 h at 100 °C. After cooling, the mixture was filtered through a fritted funnel and ethanol (1·2 volumes of 70 % v/v) was added to precipitate the glycogen. The mixture was boiled, left to cool to facilitate the flocculation of glycogen, then centrifuged (1068***g***, 5 min), and the ethanol removed with a Pasteur pipette. The glycogen was dissolved in water and reprecipitated twice with ethanol as described above before freeze drying. Samples and standards (up to 200 μg) in distilled water (800 μl) and controls (800 μl) were mixed in glass vials with 5 % (w/v) phenol (800 μl). Four ml of concentrated sulfuric acid was rapidly added to the surface of the liquid, taking care not to touch the sides of the glass vial. The mixture was left to stand for 10 min before shaking vigorously. Absorbances were determined at 490 nm after a further 30 min standing, and glycogen contents were calculated per IJ (ng IJ^{-1}).

Effect of iodoacetamide on glycogen utilization in infective juveniles of Steinernema carpocapsae

Iodoacetamide (Sigma, Poole, UK), shown to be active against nematodes by Butterworth, Perry & Barrett (1989), was used to inhibit glycogen metabolism. A total of 3×10^5 IJs in buffered distilled water (50 ml; Tris–HCl, 50 mM, pH 7·4) were added to an aerated plastic culture box ($15 \times 11 \times 6$ cm) containing 100 ml of buffered 10^{-4} M iodoacetamide (Tris–HCl, 50 mM, pH 7·4). Twelve culture boxes were incubated at 25 °C. The inhibitor solution was changed weekly and the incubation medium shaken daily. Control treatment IJs were incubated in buffered distilled water. Three samples of IJs (> 5×10^4 IJs per 4 boxes) were taken on days 0, 17, 40, 84 and 119 for the determination of glycogen (see above); the number of IJs in each sample and their dry weight were estimated before analysis. Infectivity on days 0, 40, 84 and 119 was determined using a sand-tube bioassay. Sterilized sand was moistened with 0·5 ml of sterile distilled water for every 25 ml of sand. Fifteen ml of moistened sand were placed in a 30 ml screw cap tube (2·2 diam. × 8 cm). One ml of nematode suspension (100 IJs) was transferred to a centrally-made hole in the sand (3 cm deep). A late instar *G. mellonella* larva (200–300 mg wet weight) was placed on the surface of the sand, the cap screwed on, and the tube inverted and incubated at 20 °C for 68 h. The cadavers were then dissected and the number of nematodes which had invaded counted. Each treatment was replicated 10 times.

Effect of iodoacetamide on the infectivity of aged infective juveniles of Steinernema carpocapsae

This experiment examined the role of glycogen in the infectivity of aged IJs of *S. carpocapsae* which had low levels of neutral lipid. Freshly harvested IJs were stored in distilled water at 25 °C (see above) and their neutral lipid content was monitored at regular intervals using an Oil Red O lipid index (8 = full of lipid and 1 = low lipid levels; Patel *et al.* 1997). Infective juveniles aged for 80 days, with a mean lipid index of about 1 (*ca.* 10 % of initial neutral lipid content), were used together with freshly harvested nematodes. Infective juveniles were treated in buffered iodoacetamide (10^{-3} M and 10^{-4} M) for 24 h at 20 °C. Control treatment IJs (aged and freshly harvested) were incubated in buffered distilled water. The IJs were then washed 4 times in distilled water and resuspended in distilled water at a concentration of 100 IJs/ml, and their infectivity determined in a sand-tube bioassay (see above). Each treatment was replicated 10 times. Infective juveniles remaining in the sand tubes were incubated for a further 11 days at 20 °C (without an insect), and then extracted and washed several times in distilled water. The infectivity of these IJs was determined using the sand-tube bioassay (see above). The extraction procedure recovered enough nematodes for 8 replicates per treatment; only living IJs were used in the bioassay and recovery was about 80 %.

Statistical analysis

The statistical package GLIM (© 1985 Royal Statistical Society, London; Numerical Algorithms Group, Oxford) was used for analysis and significance was tested at the 5 % level. Glycogen data were log transformed and analysed by ANCOVA. Infectivity data were analysed by ANCOVA as logits using a binomial error distribution (see Crawley, 1993).

RESULTS

Freshly harvested IJs of the 4 *Steinernema* species contained appreciable amounts of glycogen (Table 1) and utilization occurred at 2 distinct rates; 'fast' in *S. carpocapsae*, *S. riobravis* and *S. glaseri* and 'slow' in *S. feltiae* (Fig. 1). The survivorship of *S.*

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Table 1. Glycogen content (ng IJ^{-1}) of freshly harvested infective juveniles of 4 *Steinernema* species

Species	Mean (±S.E.) glycogen content (ng IJ^{-1})*
S. carpocapsae	15·0±2·3 (13·2)
S. riobravis	12·4±1·6 (8·2)
S. feltiae	35·3±2·1 (16·3)
S. glaseri	77·0±4·9 (17·7)

* Percentage dry weight of glycogen in parentheses; $n = 3$.

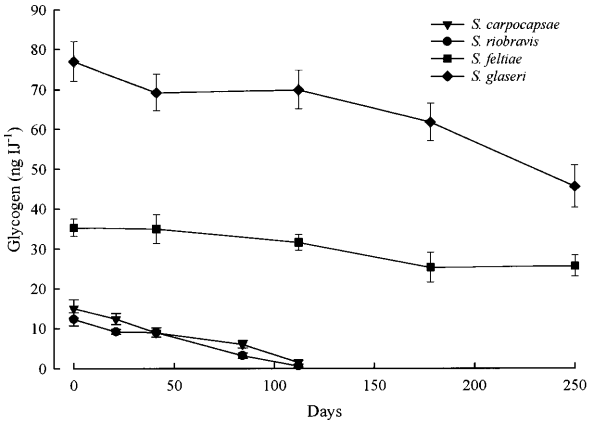

Fig. 1. Glycogen utilization (ng IJ^{-1}) in infective juveniles of 4 *Steinernema* species stored in distilled water at 25 °C. Bars = ±S.E.

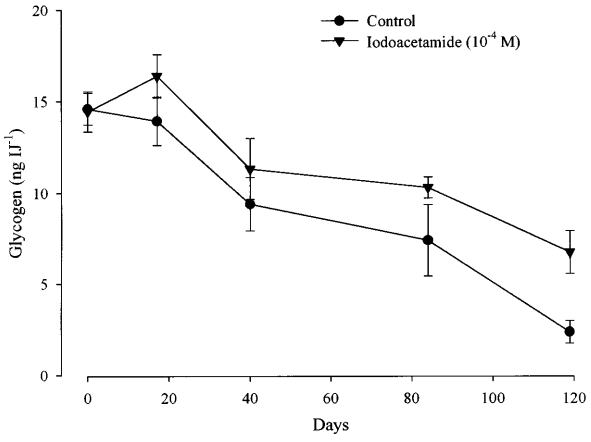

Fig. 2. Glycogen utilization (ng IJ^{-1}) in infective juveniles of *Steinernema carpocapsae* incubated with the glycolytic inhibitor iodoacetamide (10^{-4} M) at 25 °C. Bars = ±S.E.

carpocapsae and *S. riobravis* IJs ranged between 120 and 135 days, and glycogen content declined at an almost constant rate (*ca.* 0·1 ng/IJ/day) with 5–10% of the initial amount remaining after 112 days. Infective juveniles of *S. feltiae* and *S. glaseri* survived for more than 450 days, and by day 250 glycogen content had decreased by 27 and 40%, respectively.

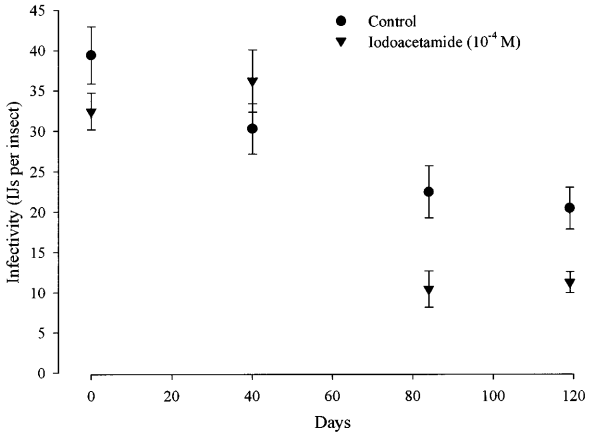

Fig. 3. Infectivity of *Steinernema carpocapsae* infective juveniles incubated with the glycolytic inhibitor iodoacetamide (10^{-4} M) at 25 °C. Bars = ±S.E.; $n = 10 \times 100$ IJs.

In the case of *S. feltiae*, the only significant decline ($P < 0·05$) in glycogen occurred between days 112 and 178, whereas in *S. glaseri* glycogen content declined significantly ($P < 0·05$) after day 178; the rate of decline for *S. glaseri* during the 250 day storage period was similar to *S. carpocapsae* and *S. riobravis*.

The addition of iodoacetamide (10^{-4} M) to the incubation medium of *S. carpocapsae* resulted in a significantly slower ($P < 0·05$) rate of glycogen utilization between days 40 and 119 compared with the controls (Fig. 2). By day 119, the mean glycogen content of inhibitor-treated IJs was more than twice that of the control nematodes. The infectivity of *S. carpocapsae* IJs incubated in iodoacetamide (10^{-4} M) was significantly reduced ($P < 0·05$) when assayed on days 80 and 119 compared with the control (Fig. 3).

Short-term incubation (24 h) of freshly harvested IJs of *S. carpocapsae* with 10^{-4} M iodoacetamide did not significantly ($P > 0·05$) affect infectivity compared with the control (Table 2). However, with 10^{-3} M iodoacetamide, infectivity was significantly reduced ($P < 0·05$). After allowing the inhibitor-treated IJs to recover for 11 days, the infectivity of these IJs was similar to that of the control ($P > 0·05$). The infectivity of aged IJs (80-day-old) treated with iodoacetamide (10^{-4} and 10^{-3} M) was significantly reduced ($P < 0·05$) compared with the control; with 10^{-3} M iodoacetamide having the significantly greater effect ($P < 0·05$). The infectivity of aged IJs treated with both doses of iodoacetamide recovered significantly ($P < 0·05$) but, in the case of IJs treated with a higher dose of iodoacetamide, infectivity did not recover to a level comparable with the control ($P < 0·05$).

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Table 2. Infectivity of freshly harvested and aged (80-day-old) infective juveniles of *Steinernema carpocapsae* determined after incubation with iodoacetamide (at 10^{-4} M and 10^{-3} M) for 24 h at 25 °C

(Non-infecting IJs were allowed to recover for 11 days before the second infectivity bioassay.)

		Mean (±S.E.) IJs per *G. mellonella**		
			Iodoacetamide concentration	
IJ age	Treatment	Control	10^{-4} M	10^{-3} M
Fresh	24 h incubation†	27·2 ± 2·7 a	23·7 ± 3·0 a	16·4 ± 1·2 b
	After recovery‡	24·1 ± 2·5 a	19·7 ± 3·1 a	22·7 ± 2·8 a
Aged (80-day-old)	24 h incubation†	15·8 ± 2·5 a	5·9 ± 1·5 b	0·3 ± 0·1 c
	After recovery‡	15·2 ± 2·3 a	20·1 ± 2·9 b	5·5 ± 0·7 c

* Means within rows followed by the same letter are not significantly different ($P > 0{\cdot}05$).
† $n = 10 \times 100$ IJs.
‡ $n = 8 \times 100$ IJs.

DISCUSSION

All 4 *Steinernema* species investigated had appreciable levels of glycogen, from about 8 % dry weight in *S. riobravis* to 18 % in *S. glaseri*. These are amongst the highest amounts reported for free-living parasitic stages of nematodes and are comparable with those found in some free-living species (e.g. adult stages of *Turbatrix aceti* and *Aphelenchus avenae*) and adult animal-parasitic nematodes (Barrett, 1976). The amounts estimated in our study for *S. carpocapsae*, *S. feltiae* and *S. glaseri* were similar to those reported by Selvan *et al*. (1993). When the total energy reserves (neutral lipid and glycogen; neutral lipid data from Patel *et al*. (1997)) in each species are considered, they all have approximately the same energy content on a percentage dry weight basis, *ca*. 40 %. However, in *S. feltiae* and *S. glaseri*, glycogen constitutes *ca*. 40 % of the total energy reserve compared with 30 and 21 % in *S. carpocapsae* and *S. riobravis*, respectively. The significance of the greater amount of glycogen in the former 2 species is not known but it could have a role in other aspects of nematode survival. In particular, glycogen has been shown to be involved in survival of some anhydrobiotic nematodes (Madin & Crowe, 1975). When freshly harvested IJs of the above species were desiccated slowly, the order of survival correlated with the relative amount of glycogen: *S. glaseri* > *S. feltiae* > *S. carpocapsae* > *S. riobravis* (Patel, Perry & Wright, 1997), and work on *S. carpocapsae* (All) has shown that glycogen levels decline during desiccation stress (Womersley, 1990).

Glycogen utilization has not been extensively studied in nematodes and data are restricted mainly to the parasitic species, *Ascaris lumbricoides* and a few free-living nematodes, in particular, *A. avenae* and *Caenorhabditis* sp. In the latter species, glycogen forms 8 and 3·3 % dry weight of the nematode, respectively, but only *Caenorhabditis* has been shown to utilize glycogen when starved aerobically (Cooper & Van Gundy, 1970). Lewis *et al*. (1995) reported that the glycogen content (% dry weight) of *S. carpocapsae*, *S. glaseri* and *Heterorhabditis bacteriophora* IJs remained constant during storage in water at 25 °C (implying glycogen declined with storage time) but provided no direct quantitative information. In the present study, we found that glycogen utilization occurred at a faster rate in *S. carpocapsae*, *S. riobravis* and *S. glaseri* compared with *S. feltiae*. In the case of *S. glaseri*, the fast versus slow distinction deviates from that reported for neutral lipid decline, where it was grouped alongside *S. feltiae* as a slow utilizer (Patel *et al*. 1997). However, since appreciable amounts of glycogen remained after 250 days in the case of *S. feltiae* and *S. glaseri*, the rate of decline may change in longer term experiments.

The present work agrees with findings in our previous study (Patel *et al*. 1997) where we reported that neutral lipid utilization correlated well with the fall in infectivity in *S. riobravis*, *S. feltiae* and *S. glaseri* but not in *S. carpocapsae*. However, it would appear that glycogen utilization can also be used as a measure of infectivity in all 4 species; since glycogen utilization correlated ($r^2 = 0{\cdot}9$) reasonably well with infectivity in *S. carpocapsae* (Fig. 4).

The importance of glycogen as an energy reserve in aged IJs of *S. carpocapsae* was confirmed in experiments using the glycolytic inhibitor iodoacetamide. The present study used 80-day-old IJs which had almost exhausted their neutral lipid

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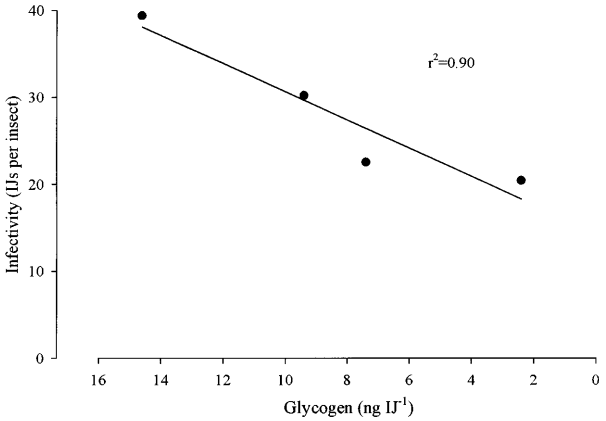

Fig. 4. Relationship between infectivity and glycogen utilization in infective juveniles of *Steinernema carpocapsae* stored in distilled water at 25 °C (control data from Fig. 3).

reserves, but the switch from infectivity relying primarily on neutral lipids to glycogen may have happened before this. The results from the long-term inhibitor incubation experiment suggest that the switch occurs after at least 40 days. However, it is important to note that 80-day-old IJs of *S. carpocapsae* still have about 10% of their initial neutral lipid content (Patel *et al*. 1997). The neutral lipid fraction mainly consists of triacylglycerols, but there are smaller quantities of other constituents in nematodes including, free cholesterol and cholesterol esters (Holz, Wright & Perry, 1997) which may not be available as a source of energy. Why the switch to a reliance on glycogen occurs in *S. carpocapsae* but not in *S. riobravis* is uncertain, particularly as the latter utilizes neutral lipid and glycogen over a similar period to *S. carpocapsae*. The current work together with our previous study on neutral lipid utilization (Patel *et al*. 1997) provides evidence that the approximate linear relationship between infectivity and energy reserves observed in *S. riobravis*, *S. feltiae* and *S. glaseri* may be the norm for nematodes of this genus. However, if we consider survival, energy utilization and infectivity in more detail, the 4 species examined fall into 3 groups: (1) *S. glaseri* and *S. feltiae*, which are long-lived (> 450 days at 25 °C), utilize total energy reserves at a relatively slow rate and show a corresponding decline in infectivity; (2) *S. riobravis*, which is short-lived (120–135 days at 25 °C), has a relatively faster rate of total energy utilization with a corresponding faster decline in infectivity; and (3) *S. carpocapsae*, which is short-lived (120–135 days at 25 °C), utilizes energy reserves at a relatively fast rate but in which infectivity is maintained at higher levels for a greater period compared with *S. riobravis*.

On a wider perspective, the groupings described above relate to the foraging behaviours observed in entomopathogenic nematodes (Lewis, Gaugler & Harrison, 1992; Campbell & Gaugler, 1993). *Steinernema carpocapsae* and *S. riobravis* have many characteristics of ambush (sit-and-wait) foragers; they spend a large proportion of their time nictating, more so in the case of *S. carpocapsae* (Campbell & Gaugler, 1993; Patel & Wright, 1996; M. N. Patel, unpublished data). While *S. feltiae* and *S. glaseri* are typical of cruising foragers. The latter strategy being more energetically expensive could account for the relatively large amounts of energy reserves present in *S. feltiae* and *S. glaseri*. However, while a biochemical and physiological understanding of entomopathogenic nematodes helps considerably in distinguishing these otherwise similar organisms, there is an urgent need to relate this information to the ecology of the nematode in its natural surroundings.

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