Neutral lipids and the assessment of infectivity in entomopathogenic nematodes: observations on four *Steinernema* species

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(*Received 4 October 1996; revised 9 November 1996; accepted 9 November 1996*)

SUMMARY

An 8-point visual index was developed for Oil Red O staining of neutral lipids in infective juveniles (IJs) of *Steinernema carpocapsae* (All), *S. riobravis* (Biosys 355), *S. feltiae* (UK76) and *S. glaseri* (NC). The visual index was found to be a reliable and rapid method for determining the relative neutral lipid content of individual IJs and was validated quantitatively by gas chromatography. The relationship between neutral lipid utilization and infectivity of IJs stored in distilled water at 25 °C was also investigated and the first quantitative results on neutral lipid utilization in entomopathogenic nematodes are reported. Neutral lipid contents of freshly harvested IJs of *S. carpocapsae*, *S. riobravis*, *S. feltiae* and *S. glaseri* were 31, 31, 24 and 26 % dry wt, respectively. *Steinernema carpocapsae* showed a sigmoidal pattern for neutral lipid utilization while *S. riobravis* used neutral lipids at an almost constant rate. Survivorship of these two species ranged between 120 and 135 days, whereas *S. feltiae* and *S. glaseri* lived > 450 days and had a slower rate of lipid utilization during a 260 day storage period. Oil Red O staining showed that individual IJs in each population utilized lipids at different rates, even though they had the same initial lipid index. The infectivity of *S. riobravis*, *S. feltiae* and *S. glaseri* declined with lipid utilization. In contrast, *S. carpocapsae* maintained a high level of infectivity even at relatively low lipid levels. Therefore, neutral lipid content was found to be a suitable indicator of infectivity for *S. riobravis*, *S. feltiae* and *S. glaseri* but not for *S. carpocapsae*.

Key words: entomopathogenic, nematodes, *Steinernema* spp., Oil Red O, neutral lipid, infectivity.

INTRODUCTION

Infective juveniles (IJs) of entomopathogenic nematodes are non-feeding and their infectivity has been shown to decline with storage time and total lipid content (Lewis *et al*. 1995). Neutral lipids (triacylglycerols) form the major energy reserve in free-living stages of a number of animal-parasitic nematode species (Lee & Atkinson, 1976) but quantitative data on this class of lipids has yet to be published for entomopathogenic nematodes.

The determination of neutral lipid content can be achieved chemically (e.g. gas chromatography), or histochemically using the stain Oil Red O (e.g. Lee, 1960; Croll, 1972). Chemical methods have the disadvantage of being time consuming and requiring large numbers of nematodes to obtain a single reading, whereas, the latter method is more rapid and can be applied to individual nematodes. Microdensitometry (e.g. Croll, 1972; Storey, 1983, 1984) and image analysis (e.g. Moens & Hendrickx, 1993; Stamps & Linit, 1995) have been used to 'quantify' the degree of Oil Red O staining in nematodes, although the need for specialist instrumentation limits the scope of these methods. Recently, a 6-point visual index has been developed for assessing the degree of Oil Red O staining in infective J2 stages of root-knot nematodes (*Meloidogyne* spp.) under field-laboratory conditions (Christophers *et al*. 1996). This simple procedure allows a large number of nematodes to be examined in a relatively short time and, because individual nematodes are being measured, provides a more complete picture about how nematodes in a population utilize their lipid energy reserves.

In the present study, we (i) developed a visual index for assessing the neutral lipid content of IJs of steinernematid nematodes, (ii) validated this index quantitatively by gas chromatography (GC) for *Steinernema carpocapsae*, *S. riobravis*, *S. feltiae* and *S. glaseri* and (iii) investigated the relationship between the decline in neutral lipids and infectivity for the above species. The paper therefore also contains the first quantitative data on neutral lipid utilization for entomopathogenic nematodes.

MATERIALS AND METHODS

Nematodes and insects

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

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https://doi.org/10.1017/S0031182096008748 Published online by Cambridge University Press

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. Infective juveniles were stored in distilled water in plastic culture boxes (15 × 11 × 6 cm; 60 boxes per species) and incubated at 25 °C (200 ml/box at 2000 IJs/ml). The 60 boxes were numbered and divided randomly into 3 groups of 20 for each nematode species. The storage water was changed weekly and the nematodes were shaken daily.

Oil Red O staining

Aliquots (2 ml) of nematodes were taken from 10 randomly chosen culture boxes for each nematode species at various intervals for 135 days for *S. carpocapsae* and *S. riobravis*, 260 days for *S. feltiae*, and 320 days for *S. glaseri*. Each sample was considered a replicate. Half the nematodes in each sample were used for infectivity studies (see below) and the other half were stained with Oil Red O using a modification of the method described by Croll (1972). A saturated solution of Oil Red O (Merck, Dorset, UK) was prepared in 70 % (v/v) ethanol. Nematodes (ca. 600) in 0·5 ml of water were transferred to a test-tube, Oil Red O (3 ml) was added and the tube incubated at 60 °C for 20 min. Excess stain was removed, 4 ml of glycerol (50 % v/v) were added, and the mixture left overnight at room temperature. Forty randomly chosen IJs per replicate were scored for lipid staining according to an 8-point lipid index (Fig. 1). A score of 8 indicated maximum lipid content while individuals with a score of 1 were considered lipid depleted.

Chemical analysis

Nematodes were sampled at various intervals for 100 days for *S. carpocapsae* and *S. riobravis*, and 260 days for *S. feltiae* and *S. glaseri* from all 60 boxes (1 pooled sample of > 100000 IJs/20 boxes) and the number of IJs in each sample estimated by dilution. The extraction of lipids was based on the technique described by Christie (1989). Nematodes were homogenized by sonication and then freeze-dried. Lipids were extracted with 19 volumes of chloroform–methanol (2 : 1 v/v) at 4 °C for 48 h. Contaminants were removed by adding 0·04 % (w/v) $CaCl_2$, corresponding to 22 % by volume of the combined solvent. The mixture was centrifuged for 10 min at 681 **g** to give 2 clear phases. The upper phase was discarded and the lower phase (chloroform) was washed with an equal volume of equilibrated solvents containing chloroform : methanol : 0·04 % (w/v) $CaCl_2$ (3 : 48 : 47 v/v). The mixture was centrifuged for 10 min at 681 **g** and the upper phase discarded. Absolute ethanol (200 μl) was added to the lower phase and the solvents removed by rotary film evaporation. The dried lipids were then dissolved in a small volume of chloroform. A small portion (10 %) was set aside for determination of total lipids and the remainder was transferred to a solid-phase chromatography column (NH_2-aminopropyl, 500 mg, 3 ml; Bond Elut®, International Sorbent Technology Ltd, Mid-Glamorgan, UK). The neutral lipids were eluted with 4 ml of chloroform : propan-2-ol (2 : 1 v/v) (Kaluzny *et al*. 1985) and dried by rotary film evaporation. The lipids were saponified and converted to fatty acid methyl esters (FAMEs) using a mixture of 1 ml of toluene and 1 % (v/v) sulphuric acid in 2 ml of methanol (Christie, 1989). The dry FAMEs were dissolved in a known volume of hexane and analysed by gas chromatography (GC) using a Varian 6000 equipped with a capillary column (Carbowax EconoCap™ 30 m × 0·32 mm internal diameter, 0·25 μm film thickness, Alltech Associates, Lancashire, UK) and a flame ionization detector (270 °C). Injections (at 250 °C) were made in split mode (50 : 1) with N_2 as the carrier gas. The temperature program was isothermal at 100 °C for 2 min, 10 °C/min to 160 °C and 2 °C/min to 235 °C and held for 1 min. FAMEs were identified by reference to authentic samples and by using the 'equivalent chain-length' concept (Christie, 1989). The addition of an internal standard (C23 : 0) allowed quantification of the FAMEs and hence the determination of total and neutral lipids. The day-to-day coefficient of variation over 16 consecutive work-days was between 1·0 % and 2·3 % for FAMEs from C16 : 0 to C20 : 4. For C22 : 0 the coefficient of variation was 3·1 % and for C24 : 0 it was 5·8 %. Total lipid and neutral lipid contents were calculated/IJ (ng/IJ) and as a percentage of nematode dry weight.

Infectivity bioassay

Infectivity was determined using a bioassay developed by Fan & Hominick (1991*a*). Sterilized sand was moistened with 0·5 ml of distilled water for every 25 ml of sand. Fifteen ml of this moistened sand was placed in a 30 ml screw cap tube (2·2 cm diameter × 8 cm). One hundred IJs in 1 ml of distilled water were transferred to a centrally made hole in the sand (3 cm deep). A late instar *G. mellonella* larva (200–300 mg wet wt) was placed on the surface of the sand, the cap screwed on, and the tube inverted and incubated at 20 °C for 72 h. The cadavers were then dissected and the number of nematodes which had invaded were counted. Each treatment was replicated 10 times.

Statistical analysis

The generalized linear modelling package GLIM v. 3.77 (© 1985, Royal Statistical Society, London;

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Table 1. Mean (±S.E.) total and neutral lipid contents (ng/IJ) of freshly harvested infective juveniles of four *Steinernema* species determined by chemical analysis (gas chromatography)

	Mean (±S.E.) lipid content (ng/IJ)†	
Species*	Total lipid‡	Neutral lipid
S. carpocapsae	44·6±2·5 (39)	34·8±0·7
S. riobravis	59·7±3·1 (39)	46·9±2·5
S. feltiae	70·4±1·4 (33)	52·2±4·3
S. glaseri	149±0·7 (34)	115±1·0

* Mean dry weight of an IJ (day 0): *S. carpocapsae* = 114 ng, *S. riobravis* = 152 ng, *S. feltiae* = 216 ng and *S. glaseri* = 435 ng.
† $n = 3$.
‡ 'Total lipid' includes the following major lipid classes: neutral lipids, free fatty acids and phospholipids. Percentage dry weight given in parentheses.

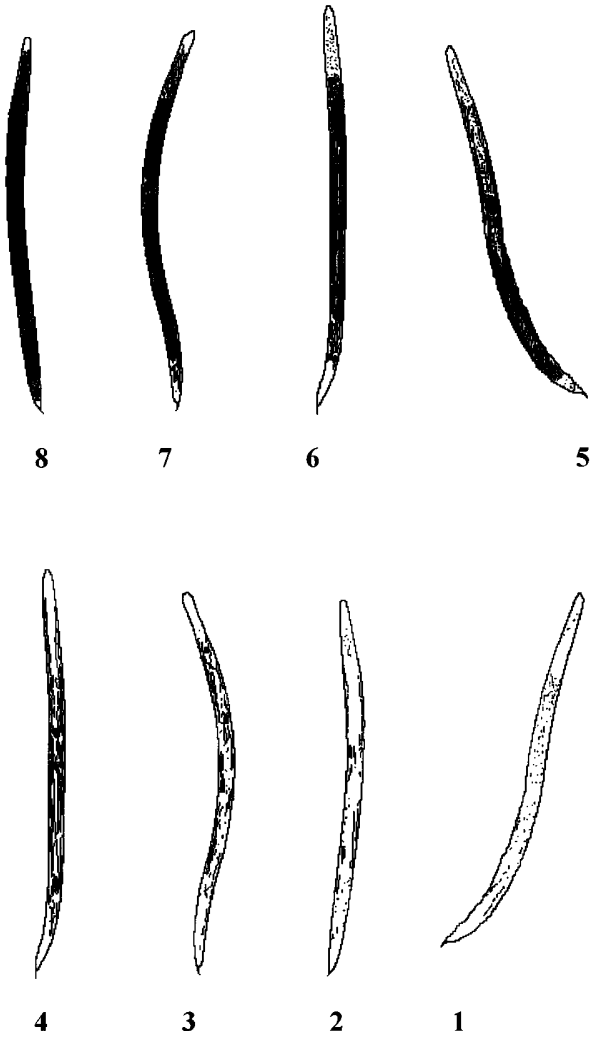

Fig. 1. Digitized images of infective juveniles stained with Oil Red O (shaded areas). Nematodes with a lipid index value of 8 are full of lipid while those with an index value of 1 are classified as lipid depleted.

Numerical Algorithms Group, Oxford) was used for statistical analysis (see Crawley, 1993). Neutral lipid determinations (by Oil Red O and quantitative chemical analysis) were subject to ANOVA. Infectivity data were analysed by ANOVA as logits using a binomial error distribution; overdispersion in the data was dealt with using an empirical scale parameter(s) calculated by dividing the Pearson χ^2 by the degrees of freedom (Crawley, 1993). Changes in deviance were tested using F-tests. Significance was tested at the 5% level.

RESULTS

The initial level of total lipid ranged between 33 and 39% of the nematode dry weight (Table 1), with *S. carpocapsae* and *S. riobravis* having the greatest amount of total lipid (39% dry wt). Neutral lipids formed between 74 and 77% of the total lipids in the 4 species.

The 8-point lipid index (Fig. 1) and the GC-based analysis of neutral lipids showed similar patterns with time of storage in the 4 nematode species (Fig. 2). The times taken for 50% depletion of lipid in *S. riobravis* were similar when measured by Oil Red O and GC. With *S. carpocapsae*, *S. feltiae* and *S. glaseri*, the 50% depletion time was consistently less with Oil Red O than with GC analysis (Table 2).

The rates of neutral lipid utilization occurred at species-specific rates (Fig. 2). *Steinernema carpocapsae* and *S. riobravis* utilized their neutral lipids over a period of about 120 days (Fig. 2A, B) while *S. feltiae* and *S. glaseri* still had appreciable levels of lipid after 260 days in storage (Fig. 2C, D). Survivorship of *S. carpocapsae* and *S. riobravis* ranged between 120 and 135 days while *S. feltiae* and *S. glaseri* survived in excess of 450 days. Based on GC analysis, *S. carpocapsae* appeared to show a sigmoidal pattern for neutral lipid utilization and the greatest rate of decline was between days 30 and 60 (Fig. 2A), whereas *S. riobravis* utilized neutral lipid at an almost constant rate between days 0 and 100 (Fig 2B). *Steinernema feltiae* and *S. glaseri* showed a far slower rate of neutral lipid utilization than the above species (neutral lipid content did not change significantly ($P < 0{\cdot}05$) during the first 80 days) (Fig. 2C, D). The pattern of neutral lipid utilization for *S. feltiae* and *S. glaseri* appeared to represent the first 2 phases of a sigmoidal curve. Neutral lipid declined at a slower rate in *S. glaseri* compared with *S. feltiae* ($P < 0{\cdot}05$) and after 260 days *S. glaseri* still had approximately 50% of its initial lipid level compared with approximately 30% for *S. feltiae*.

Oil Red O staining showed that individual IJs within each nematode population utilized lipids at different rates (Fig. 3). Greater than 95% of freshly harvested IJs had a lipid index of 8 but with time the IJ population of each *Steinernema* species became heterogeneous.

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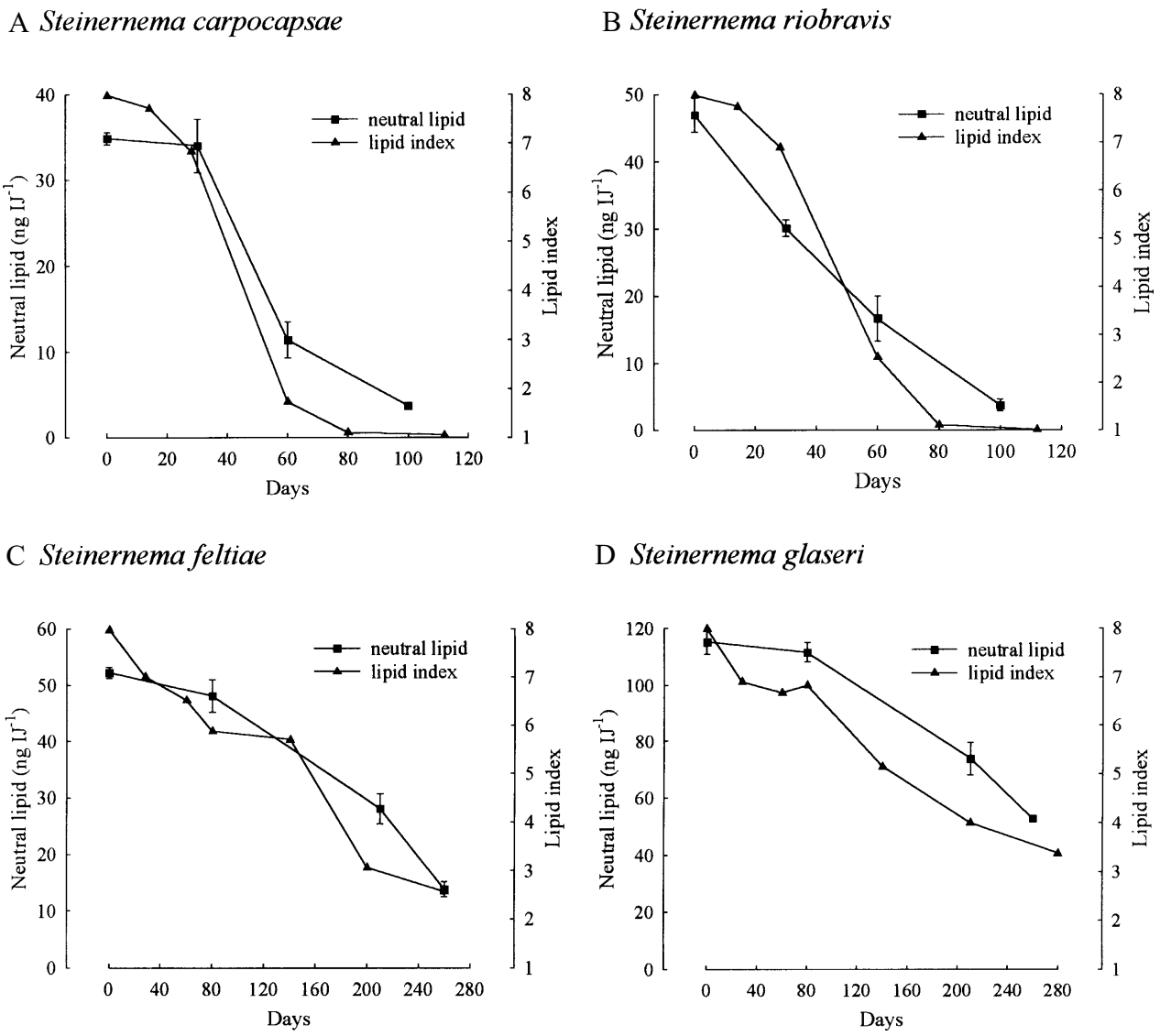

Fig. 2. Mean neutral lipid content (±S.E.) of infective juveniles of (A) *Steinernema carpocapsae*, (B) *S. riobravis*, (C) *S. feltiae* and (D) *S. glaseri* determined by quantitative chemical analysis (■) ($n = 3$) and using the Oil Red O lipid index (▲) ($n = 10 \times 40$ IJs). Infective juveniles were stored in distilled water at 25 °C for up to 280 days.

Table 2. Fifty percent neutral lipid depletion times (days) estimated using an Oil Red O lipid index and chemical analysis for four *Steinernema* species (see Fig. 1)

	50% lipid depletion times (days)	
Species	Oil Red O Lipid index	Chemical analysis*
S. carpocapsae	44	52
S. riobravis	47	48
S. feltiae	175	212
S. glaseri	204	255

* Gas chromatography.

The initial infectivity of the 4 *Steinernema* species ranged between 30 and 60% (Fig. 4). In the case of *S. riobravis*, *S. feltiae* and *S. glaseri*, infectivity declined as the IJs utilized their neutral lipid reserves. Whereas, *S. carpocapsae* IJs maintained high levels of infectivity even at relatively low levels of lipid and infectivity only declined significantly ($P < 0{\cdot}05$) when the mean lipid index was less than 2.

DISCUSSION

The Oil Red O lipid index was found to be a reliable and rapid method for determining the relative neutral lipid content of individual IJs which, in general, correlated well with quantitative determination of neutral lipids. Where the assessment by the lipid index differed appreciably with the quantitative estimates, the lipid index usually underestimated the relative neutral lipid content. For *S. carpocapsae* and *S. riobravis* this underestimation was found when neutral lipid levels were low and may have been due to the difficulty in assessing low

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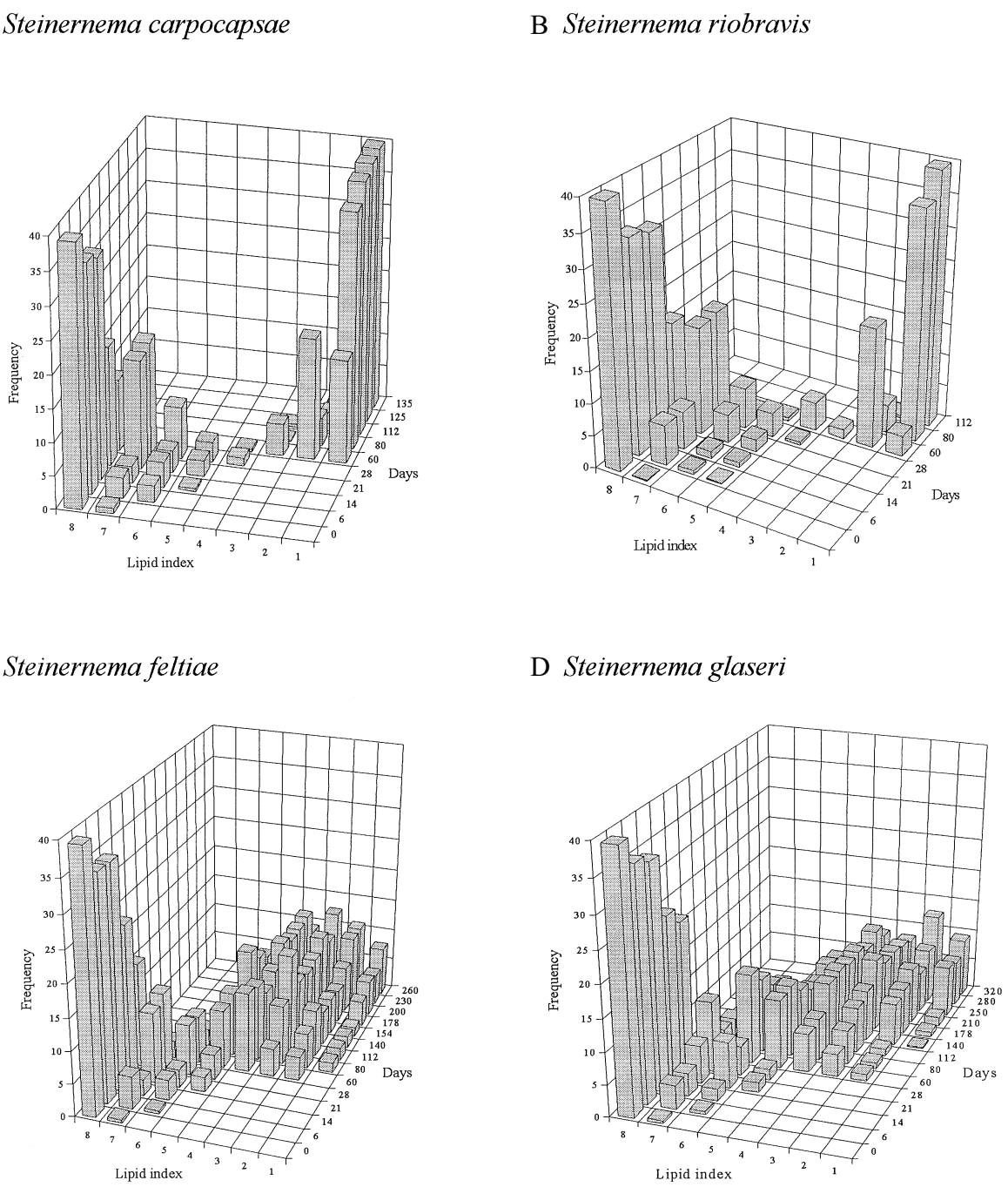

Fig. 3. Neutral lipid index scores of individual infective juveniles of (A) *Steinernema carpocapsae*, (B) *S. riobravis*, (C) *S. feltiae* and (D) *S. glaseri* stored in distilled water at 25 °C for up to 320 days ($n = 10 \times 40$ IJs).

levels of staining in these relatively small nematodes ($\leqslant 650\,\mu$m). The relatively larger size of *S. feltiae* and *S. glaseri* IJs ($\geqslant 800\,\mu$m) made visual assessment easier although the consistent underestimation of the neutral lipid content in *S. glaseri* using the Oil Red O lipid index may have been due to the greater diameter of *S. glaseri* (greatest diameter = 43 μm; almost twice that of *S. feltiae*), making it more difficult to visually detect stained lipid through the body of the nematode.

A visual index has three major advantages compared with conventional microdensitometry or image analysis. Firstly, it can be used easily in a non-specialist laboratory. A similar lipid index developed for *Meloidogyne* spp. has been used successfully in field laboratories (Christophers *et al.* 1996). Secondly, it provides a rapid and cheap way of processing a large number of samples. Thirdly, individual nematodes can be assessed, therefore providing information on the distribution of neutral lipid in a nematode population. We consider the latter to be an important attribute as currently there are no methods available for rapidly generating such information. The present study showed how heterogeneous the IJ population of each species became for neutral lipid during storage in water; indicating

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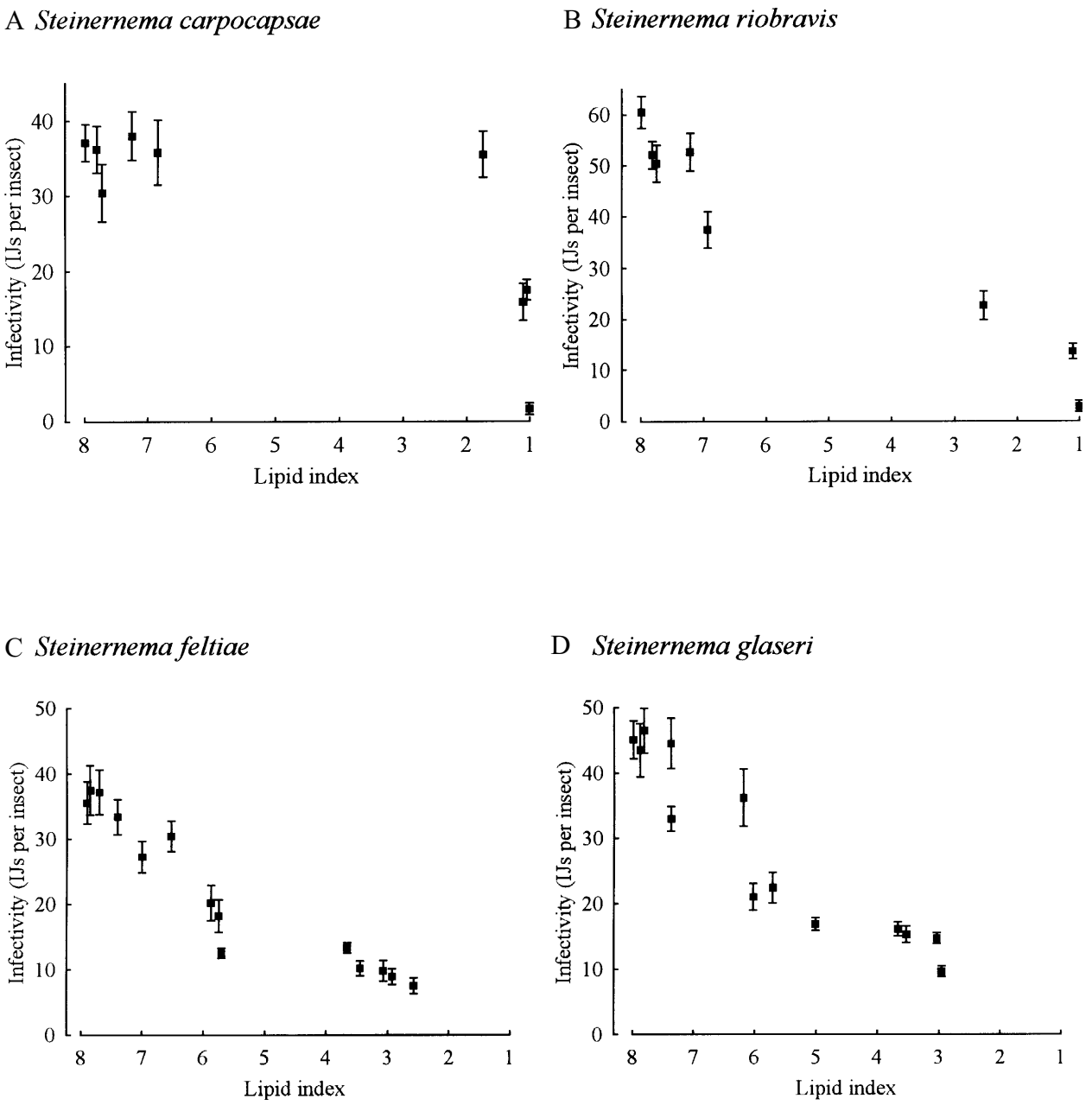

Fig. 4. Relationship (±S.E.) between infectivity and mean neutral lipid index scores of infective juveniles of (A) *Steinernema carpocapsae*, (B) *S. riobravis*, (C) *S. feltiae* and (D) *S. glaseri* ($n = 10 \times 100$ IJs for infectivity and $n = 10 \times 40$ IJs for Oil Red O staining).

individual IJs utilize lipid at very different rates. This would suggest that the locomotory activity of the IJs differed during storage or that some individuals were able to enter periods of quiescence (Evans & Perry, 1976); thus reducing lipid utilization. While it is widely known that IJs of some entomopathogenic nematode species, including *S. carpocapsae* and *S. riobravis* readily adopt a stationary straight posture when stored in water, IJs of *S. feltiae* and *S. glaseri* were rarely observed to be inactive during daily inspections in the present study. Storey (1984) also found lipid utilization to be variable between individual juveniles of *Globodera rostochiensis* and attributed this to genetic variability even though the nematodes were derived from a small founder population. Heterogeneity in lipid utilization would serve as a useful population survival strategy for entomopathogenic nematodes, particularly since individual IJs produced within a single insect host are likely to be genetically similar.

The estimates of total lipid content produced in the present study agree well with those produced by Selvan, Gaugler & Lewis (1993) for *S. carpocapsae*, *S. feltiae* and *S. glaseri* (they did not look at *S. riobravis*). Our profiles for neutral lipid utilization differ from those obtained by the same workers for total lipid decline in *S. carpocapsae* and *S. glaseri* during storage in water at 25 °C (Lewis *et al.* 1995).

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However, the duration over which lipid was utilized in our study was similar. These studies suggest that IJs of entomopathogenic nematodes can be classed as either fast or slow with regard to their rate of lipid utilization. Although only a few steinernematid species have been studied, the size of the IJ and the amount of initial neutral lipid content appears to correlate well with survivorship and the rate at which the nematode will utilize lipids; species with relatively small IJs (e.g. *S. carpocapsae* and *S. riobravis*) utilize lipids faster compared with those with larger sized IJs (e.g. *S. feltiae* and *S. glaseri*). The distinction between fast and slow lipid utilizing species is a general one since it is clear from the lipid index distributions that within the populations of the 'slow' species, *S. feltiae* and *S. glaseri*, there were individual IJs which used their neutral lipid reserves at similar rates to some of the 'slowest' utilizing individuals in the 'fast' species, *S. carpocapsae* and *S. riobravis*.

In the present work, infectivity was found to be related directly to lipid content in the case of *S. riobravis*, *S. feltiae* and *S. glaseri*. However, *S. carpocapsae* did not show this relationship and infectivity declined only when the lipid index had fallen below 2. Additional studies have also shown a lack of correlation between lipid levels and infectivity, and have suggested that *S. carpocapsae* can utilize glycogen when lipid levels are low (mean lipid index of about 1) to maintain infectivity (M. N. Patel & D. J. Wright, unpublished data).

Storage time alone is not a useful indicator of the infectious status of IJs of entomopathogenic nematodes since differences in storage conditions, substrates and the ability of nematodes to remain inactive are also important considerations. For example, storage below 10 °C can produce U-shaped infectivity profiles (Fan & Hominick, 1991*b*) or result in increased infectivity (Ishibashi, Wang & Kondo, 1994). Therefore, lipid utilization would provide a better measure of physiological aging than time alone.

The present study has shown that (i) lipid utilization is a more appropriate covariate than age for assessing infectivity in 3 species of commercially produced steinernematid nematodes, (ii) the use of a simple lipid staining index provides a rapid and powerful means of assessing the neutral lipid content of individual nematodes, and (iii) neutral lipid content is not a suitable indicator of infectivity for *S. carpocapsae*.

We thank B. Briscoe (CAB International Institute of Parasitology, St Albans, UK) for providing the initial nematode cultures, and Dr F. G. P. E. Earley (Zeneca Agrochemicals, Jealott's Hill, Bracknell, UK) for the donation of the GC equipment. M. N. Patel was supported by a Biotechnology and Biological Sciences Research Council (UK) CASE Studentship, Biosys Inc. (Columbia, Maryland, USA) and CAB IIP. M. Stolinski was supported by a Science and Engineering Research Council (UK) CASE Studentship and Zeneca Agrochemicals.

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