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THE USE OF NEMATODES AS A REPLACEMENT FOR *ARTEMIA* AND ROTIFERS IN THE AQUACULTURE INDUSTRY

A THESIS SUBMITTED TO THE UNIVERSITY OF WALES

BY

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SUMMARY

The objective of this research was to find a nematode species that could replace the use of *Artemia* and rotifers as a fish and shrimp feed in aquaculture hatcheries. *Panagrellus redivivus* is shown to be the most promising nematode of all the species tested. An inexpensive medium containing bovril, yeast extract and corn oil/fish oil was developed for the culture of this species. It is demonstrated that *P. redivivus* can be mass produced using conventional fermentation technology and formulated into a carrier matrix such as sodium alginate gel.

Present research demonstrates that the fatty acid profile of the nematode can be manipulated by the addition of fish oil rich in highly unsaturated fatty acids (HUFAs) to the growth medium of the nematode. This produces an increase in the essential fatty acids (EFAs) such as eicosapentaenoic acid (20:5ω-3) and docosahexaenoic acid (22:6ω-3). It is also shown that *P. redivivus* can be pigmented with astaxanthin by the addition of carophyll pink to the growth medium. The overall nutritional profile of *P. redivivus* was modified to be similar to that of *Artemia.*

Fish and shrimp trials undertaken using *P. redivivus* alone revealed that it could not support the growth and development of *Scopthalmus maximus, Pleuronectes platessa, Ctenolabrus rupestris* and *Clupea harengus*. However nematodes pigmented with astaxanthin and enriched with HUFAs support good growth and survival of *Penaeus indicus* larvae.

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Chapter 5 - Preliminary shrimp and larval growth trials.

Chapter 6 - Shrimp larval growth trials on lipid enriched and pigmented nematodes.

Chapter 7 - Preliminary fish trials.

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INTRODUCTION

INTRODUCTION

Farmed shrimp production particularly in Asia and South America is growing rapidly. Overall shrimp production in 1965 increased from 0.7 million tons to 2.5 million tons in 1990 and may exceed 3.2 million tons by the year 2000 (Csawas, 1994). The Food and Agriculture Organisation of the United Nations (FAO) reported volumes of farmed shrimp reaching 884,075 tons in 1992 (Cited in Fish farming 1994) making this 30% of the global shrimp supply in 1992 (Csawas, 1994; Landsman, 1994). The shrimp industry provides one of the major sources of income in developing countries eg. Ecuador, Bangledesh, Phillippines (Landesman, 1994). The major culture species are *P. monodon* (49.8%), *P. chinensis* (13.8%) and *P. vannamei* (15.5%) (Csawas, 1994). *P. indicus* is cultured extensively in shrimp farms throughout Southeast Asia, mainly in the Philippines and constitutes 5.4% of the total farm raised shrimp production in 1992 (Weidner and Rosenberry, 1992).

For many years shrimp farms have supplied their seed requirement from wild caught postlarvae. However wild seed stocks are limited and cannot meet the demand of the fast growing industry that needs a continuous supply of post larvae throughout the year. Hatcheries have been established to meet these demands of the shrimp industry over the last few decades. These hatcheries rely on wild collected gravid females for the production of shrimp nauplii. Hatching management requires a proper water quality control and appropriate feeding regimes. In large well equipped hatcheries, seawater used in larval culture is generally filtered and UV treated to prevent disease breakout.

The larval stages of marine aquaculture organisms, including both penaid prawns and fish such as turbot also require a live feed with a suitable nutritional composition under natural conditions. This can cause problems for hatcheries in terms of ensuring a regular supply of good quality feed. At present, penaeids are fed with microalgae, grown on site, weaned onto the brine shrimp *Artemia* and then fed pelleted or flake feeds. Marine fish larvae are either fed on rotifers, which again are grown on site or if large enough with *Artemia*.

Table 1. The use of a mixed algal diet for shrimp larvae.(After Jones et al.,1993).

Species	Feed type	Density	Stage	Survival %	Author
P. aztecus	*Skeletonema costatum* *Artemia*	6-10 x10^3 cells 10 nauplii	Z1-M1, Z3-PL1	50.0	Cook (1967)
P. indicus	*Thalassiosira weisflogii*	4x10^3 cells 7x10^3 cells 2x10^3 cells	N6-PZ1 PZ1-PZ3 PZ3-M3	95.0	Emmerson (1980, 1984)
P. indicus	*Chaetoceros gracilis (40%)/* *Platymonas sp. (40%)/ Isochrysis* *aff. galbana (20%)* *Artemia*	30-40x10^3 cells 1-2 nauplii	PZ1-PZ3 M1-PL1	61.0 60.0	Galgani and Aquacop (1988)
P. japonicus	*C. gracilis* *Artemia*	50-125x10^3 cells 3-7 nauplii	PZ1-PL1 M1-PL1	46.0 75.7	Rodriguez et al., (1994)
P japonicus	*C. gracilis* *Artemia*	50-125x10^3 cells	PZ1-PL1 M1-PL1	79.8-84	Le Vay et al., (1993)
P. kerathurus	*S. costatum* *Brachionus plicatilis* *Artemia*	50-100x10^3 cells	PZ1-PZ3 M1-M3 PL1-PL3	72-89	Yufera et al., (1984)
P. marginatus	*Chorella sp.* *Artemia*	250-300x10 cells 1-3 nauplii	PZ1-PL8 PZ3-PL10	60.0 58.0	Gopalakrishnan (1976)
P. monodon	*Tetra selmis chuii* *Rhinomonas reticulata* *T. chuii / R. reticulata (1:1)* *Artemia*	45x10^3cells 45x10^3cells 45x10^3cells 5 nauplii	PZ1-PL1	66.15 66.21 63.49	Kumarly et al. (1989a)
P. monodon	*T. chuii / R. reticulata (1:1)* *Artemia*	40x10^3 cells 5 nauplii	PZ1-M1 M2-PL1	86.0	Jones et al., (1989)
P. monodon	*T. chuii* *C. calcitrans* *Artemia*	50-100x10^3 cells 50-100x10^3 cells 2-5 nauplii	Z1-M3 Z1-M3 M2-M3	50.2 47.1	Tobias-Quinitio and Villegas (1982)
P. vannammei	*S. costatum / C. gracilis (1:1)* *Artemia*	140x10^3 cells 3 nauplii	PZ1-PL1 M1-PL1	88.8 88.8	Wilkenfeld et al., (1984)
P. setiferus	*S. costatum / C. gracilis* *Artemia*	140x10^3 cells 3 nauplii	PZ1-PL1 M1-PL1	91.8 93.8	Wilkenfeld et al., (1984)
P. stylirostris	*S. costatum / T. fluvitalis (1:2.5)* *Isoschrysis sp. / T. chuii (1:2.5)*	140x10^3 cells 140x10^3 cells	PZ1-M1 PZ1-M1	99.0 96.0	Kuban et al., (1985)

LIVE FEEDS.

Penaeid larvae have a non feeding stage called a nauplius which has 5-6 stages. The larva pass through 3 protozoeal stages (PZ1-3) followed by 3 mysis stages (M1-3) before reacing the postlarval stage (PL). Penaeid shrimp larvae in hatcheries are reared on microalgae (diatoms, flagellates, etc.) during zoeal stages. They are then fed zooplankton (*Artemia*, rotifers) during later stages (Hudinaga, 1942; Aquacop, 1983; Liao et al., 1983). Production of live diets at a commercial scale is complicated, expensive and unreliable in supply and nutritional value (Soorgeloos et al., 1983; Langdon et al., 1985; Jones et al., 1993). *Artemia* is the most practical animal prey, but limited resources, high cost of the cysts and nutritional variability are the disadavantage of this live food source (Sorgeloos, 1980; Watanabe et al., 1983; Leger et al.,1985, 1986). The use of a mixed algal diet for penaeid shrimp larvae always gives superior survival, growth and development to a single algal species (Kuban et al., 1985; Amjad, 1990) due to their more balanced nutrient content (Table1).

The search for a cheaper more nutritionally adequate and practical larval feed source has been directed towards other live zooplankton and artificial diets. Formally live zooplankton, rotifers (*Brachionus plicatilis*) were extensively used to feed penaeid larvae (Liao et al,1983, Yufera et al, 1984). However use is limited due to the difficulties in culture and poor nutritional quality ie. in the lack of Highly Unsaturated Fatty Acids (HUFA) (Watanabe et al., 1983). Live free living nematodes such as *Panagrellus redivivus* were suggested as a potential alternative to live feed source to replace *Artemia* in the culture of several penaeid species (Wilkenfeld et al., 1984; Biedenbach et al., 1989). Most nematodes can be cultured easily in a cheap growth medium in large quantites and it has been suggested that their nutritional value can be modified by loading the alimentary canal with growth factors such HUFA (Kahan et al, 1980; Rouse et al., 1992).

Of all the possible live foods that are available to fish and shrimp larvae, *Artemia* has been found to be suitable food for the most diversified groups of oganisms of the animal kingdom, eg. foraminifers, coelenterates, flatworms, polychaetes, squids, insects, chatognaths and a wide variety of both marine and freshwater crustaceans and fishes (Sorgeloos, 1980). Success is mainly due to the ease of culture and nutritional content. The first significant advancement in

the use of *Artemia* was made in the USA (Seal, 1933) and Norway (Rollefsen, 1939). They discovered that the nauplius stages of the larval *Artemia* makes an excellent food source for shrimp and fish larvae. The main advantage in using *Artemia* is due to the dry cysts formed which are inactive embryos. These cysts remain viable for up to a year making them easy to transport and for this reason they are widely used commercially. The hatching of the cysts is initiated by incubating the cysts in salt water for up to 24h to produce free swimming larvae. *Artemia* have been successfully used for rearing the larval stages of a variety of shrimp, in particular *Macrobrachium* and *Penaeus* sps, which depend on *Artemia* for a long period of time in their development (Bledsoe et al., 1978).

Several different strains of *Artemia* were soon recognised (Watanabe et al., 1978, 1979; Fujita et al., 1980). Watanabe et al., (1978) analysed *Artemia* cysts and nauplii from different locations and it was demonstrated that *Artemia* could be classified into two types by their fatty acid composition, one contained a high amount of linolenic/octadeca-9,12,15-trienoic acid (18:3ω3) which is an essential fatty acid (EFA) for freshwater fish and the other was high in the content of eicosapentaenoic acid (20:5ω3) and docosahexaenoic acid (22:6ω-3) which were EFAs for marine fish (Watanabe et al., 1978,1993). Watanbe et al., (1980) clearly demonstrated that the content and class of fatty acid contained in *Artemia* are the principal factors in the food value of *Artemia* fed to fish and shrimp. Red sea bream juveniles (*Pagrus major*) showed a high mortality when fed freshwater *Artemia*. However there was a high survival rate when juveniles were fed *Artemia* cultures from a marine type environment. When the fatty acid profile of *Artemia* was analysed it was found to vary from place to place and sometimes differed from year to year (Fujita et al., 1980). *Artemia* cysts from different localities (San Francisco, Canada and China) were analysed for their fatty acids composition of EFAs and differences were also observed between the batches.

Artemia produced in different locations showed a similar mineral composition regardless of where they came from. Roeder and Roeder, (1966) reported that a low dietary value of *Artemia* was due to the low iron which was observed to be several times higher in Artemia from South America and Canada (Watanbe et al., 1983). Results of a detailed characterization study of five selected strains of *Artemia* are reported by {Johns and Walton, (1979); Beck et al, (1980); Johns

et al.,(1980); Klein-Macphee et al., (1980); Olney et al., (1980); Schauer et al., (1980) and Seidal et al., (1980)} cited in Sorgeloos, (1980).

Matsuoka (1975) reported that Chinese *Artemia* were unable to support the growth and development of *Macrobrachium rosenbergii* larvae. Pesticide analysis revealed 5 to 10 times higher concentrations of DDT in Chinese *Artemia* compared to San Francisco Bay *Artemia.* A bad performance of *Artemia* was also reported from Great Salt Lake and it was thought that residual pesticides accumulate in the *Artemia* (Slobodkin, 1968) and that *Artemia* produce an immunity to these alkaloids by concentrating them in the cysts (Provasali, 1969) cited in Sorgeloos , (1980). Alternatively it was thought that there might be a mineral deficiency in the lake (Oppenheim, in Provasili, 1969). Much effort has been made to reduce the amount of pesticides in *Artemia.* As a result contaminants such as DDT are no longer a problem in the 1990's. However the source of supply of *Artemia* has become a major problem for the aquaculture industry (Sorgeloos, 1983). Harvests from natural lakes have fluctuated drastically in recent years producing shortages and high prices. Since Biedenbach et al., (1989) demonstrated the use of nematodes as a suitable replacement for *Artemia*, there has been a limited use of nematodes in some penaeid hatcheries, but their application is limited by difficulties of supply and cost effectiveness. The objective of this research is to identify suitable nematodes species for culture and to test their nutritional quality for marine, fresh water fish and penaeid shrimp larvae. Present work also aims to culture suitable nematodes species on a large scale and to modify the profile of nematodes for use in commercial hatcheries.

THE USE OF NEMATODES WITH SHRIMP LARVAE.

The raising of shrimp and fish on a commercial scale has been synonymous with the problem of mass producing a suitable food organism. It was the suggestion by Bolin in (1932) cited in Seal, (1933) that *Artemia salina* was utilised which proved a great success, but has been plagued with problems as previously discussed. Aquaculturists are always looking for an alternative, more efficient and reliable method for rearing larvae and as long ago as (1939) Plagman suggested that nematodes could be a possible food source for shrimp. Single nematodes were found in the stomach of shrimp of various sizes (11-75mm) and in one shrimp more than 15 nematodes were

found. The shrimp *Crangon crangon* of different size classes were found to survive for periods of up to 210 days when fed exclusively with free living nematodes, and were observed to catch the nematodes from the sandy substratum. The food intake was much less than it appeared and it was observed that combination of meiofauna and nematodes was best. Smaller shrimp had a better survival than larger shrimp when fed on nematodes. In the absence of other foods it was thought that nematodes provided a good supplement and prevented starvation (Gerlach and Scharge, 1971).

The present rearing techniques for larval penaeids relies on a complex chain of live foods (Hudinaga, 1967; Cook and Murphy, 1969; Mock and Neal, 1974; Shigueno, 1975; Platon 1978 and Liao 1984). Pioneering work by (Hudinaga, 1942) developed a regime that required a unicellular algal source during the protozoeal substages and then an animal feed (*Artemia*) during the mysis substages. This has been universally followed although the risk of a culture collapse is quite high. Although the ultimate goal of larviculture research is to develop artificial formulations for penaeids that will replace live foods (Fontaine et al., 1982; Quinitio et al., 1983) alternative sources of live feeds to replace algal and animal feeds is also under examination.

Wilkenfeld et al (1984) performed feeding experiments on the following species; *Penaeus aztecus*, *P. setiferus* and *P. vannamei* of shrimp larvae using nematodes. Survival, metamorphosis, dry weight and growth of the larvae were measured and were fed a variety of algae and animal food regimes. The objective was to compare the nematode *Panagrellus redivivus* and *Artemia* as animal food sources. A comparison was made between feeding the diatoms *Skeletonema costatum* and *Chaetoceros gracilis*, pytoflagellates *Isochrysis sps.* and *Tetraselmis chuii* with nematodes or *Artemia* nauplii added to the diet starting at the protozoea II or mysis I substage. The data presented in this paper (Tables 2 and 3) indicated that the nematodes could replace part or all of the *Artemia* in a larval feeding regime, producing a survival and dry weight growth equal to that attained by larvae fed *Artemia* nauplii. However the techniques used to grow nematodes could not be scaled up and transport methods for nematodes were unknown.

Table 2. Metamorphosis of *Penaeus aztecus*, *P. setiferus* and *P. vannamei* larvae to the postlarvae-one (PL1) substage when fed *Panagrellus redivivus* or *Artemia* nauplii in combination with diatoms or Phytoflagellates starting at the protozoea-two (P2) or mysis-one (M1) substage. After Wilkenfeld et al., (1984).

Treatment	% Metamorphosis to postlarva-one +/- S.D.		
	Penaeus aztecus	*Penaeus setiferus*	*Penaeus vannamei*
S. costatum/C. gracilis only	90.8+/-3.8	90.4+/-4.0	45.6+/-5.4
S. costatum/C. gracilis P. redivivus P2	94.8+/-3.4	96.0+/-2.4	72.4+/-10.4
S. costatum/C. gracilis Artemia P2	95.2+/-3.0	95.8+/-3.3	84.6+/-2.1
S. costatum/C. gracilis P. redivivus M1	93.8+/-6.3	95.6+/-1.5	65.2+/-6.1
S. costatum/C. gracilis Artemia M1	93.0+/-6.1	95.4+/-2.3	74.0+/-6.3
Isoschrysis/T. chuii only	0.0 +/-0.0	33.2+/-29.3	-
Isoschrysis/T. chuii P.redivivus P2	57.4+/-12.7	75.6+/-5.0	-
Isoschrysis/T.chuii Artemia P2	92.6+/-2.3	84.6+/-5.0	-
Isoschrysis/T.chuii P. redivivus M1	20.2+/-15.6	77.4+/-6.1	-
Isoschrysis/T.chuii Artemia M1	50.8+/-11.5	87.4+/-4.0	-
P.redivivus only	1.2+/-2.2	0.6+/-1.3	0.0 +/-0.0
Artemia only	0.0+/-0.0	0.0+/-0.0	-

ARTIFICIAL DIETS

Attempts to reduce the dependence of fish and crustacean larvae on an exclusively live diet have gained some progress by introducing fresh, frozen and artificial diets (Kahan, 1984). In an ideal world larval culture would simply consist of adding appropriate amounts of pre-prepared dried feeds to the larval culture, and after a suitable period, harvesting seed for on grow (Jones et al.,1993). Over the past decade the use of artificial feeds has increased and a wide range are available in the form of encapsulated and microparticulate feeds (Table 4a and 4b).

Table 3. Mean dry weights of individual *Penaeus aztecus, P. setiferus* and *P. vannamei* at the termination of experiments in which they were fed *Panagrellus redivivus* or Artemia nauplii in combination with Diatoms or Phytoflagellates starting at the protozoe-two (P2) or mysis-one (M1) substage.

Treatment	Termination mean dry weights, µg +/-S.D.		
	Penaeus aztecus	*Penaeus setiferus*	*Penaeus vannamei*
S. costatum/C. gracilis only	71.72+/-2.76	59.39+/-6.84	61.08+/-0.97
S.costatum/ C. gracilis P.redivivus P2	104.62+/-2.49	107.64+/-1.33	95.14+/-4.90
S.costatum/C. gracilis Artemia P2	109.94+/-0.84	108.94+/-0.62	107.17+/-3.09
S. costatum/C. gracilis P.redivivus	104.76+/-2.35	104.74+/-3.55	82.88+/-3.24
S.costatum/C. gracilis Artemia M1	110.13+/-4.71	108.92+/-3.82	96.85+/-2.56
Isochrysis/T. chuii only	48.64+/-5.14	45.27+/-2.54	-
Isochrysis/T. chuii P.redivivus P2	91.39+/-2.59	87.98+/-3.52	-
Isochrysis/T. chuii Artemia P2	109.56+/-6.38	107.66+/-2.13	-
Isochrysis/T. chuii P.redivivus M1	86.42+/-1.24	91.25+/-3.83	-
Isochrysis/T. chuii Artemia M1	89.54+/-1.71	104.54+/-2.77	-
P. redivivus only	60.93+/-0.72	61.92+/-3.80	33.08+/-3.02
Artemia only	43.79+/-6.51	28.35+/-7.52	

In most cases for larval shrimp culture, artificial feeds are still fed only as partial replacements in hatcheries (50-70%). Total replacement of algae is possible with micoparticulate feeds (Kanazawa, 1990), but in general growth is significantly lower than on live feed controls. It has been demonstrated (Liao et al., 1988; Jones et al., 1989) that the lack of water stability in most microparticulate diets leads to rapid leaching, Jones et al., (1989), bacterial build up and water pollution. There has been no successful total replacement for live feeds for caridean prawn or homarid lobster larval culture. Few fish species eg. salmonids have been successfully reared from first feeding exclusively on an artificial diet. In general fresh water fish larvae are fairly large at hatching (12-25mm) and adapt to feeds relatively easily. Marine larvae have small yolk

Table 4a. Summary of live feed replacements in commercial larval crustacean culture. (After Jones et al., 1993).

Species	Feed replacement	Result
Penaeid shrimp		
P. vannamei	microcapsules + algae + *Artemia* 3-5 ml $^{-1}$	90% survival to PL5-7 (2 t tank) Jones et al. (1987).
P. vannamei	microcapsules + algae no *Artemia*	80% survival to PL5-7 (25 T tank) Jones et al. (1987).
P. monodon	microcapsules + 10 cell μl^{-1} algae	76% survival. growth same as live fed control (2L flasks) Jones et al. (1989).
P. stylirostris	microcapsules + *Artemia*	growth and survival comparable with live feeds (commercial scale) Ottogali (1991).
P. japonicus	microparticulate	75% survival (15 t tank) Kanazawa (1990).
P. indicus	microparticulate	up to 62% survival to mysis I. Growth less than algae Galgani and Aquacop (1988).
Caridean shrimp		
Macrobrachium rosenbergii	egg custard, fish, clam, shrimp partial replacement *Artemia*	77% survival to PL Ang and Cheah (1986)
M. rosenbergii	freeze dried catfish *Artemia* stage I-V	11% survival to PL Sick and Beaty (1975).
M. rosenbergi	microcapsules from stage VI	84% survival similar to control, growth 1 day slower Deru (1990).
Lobster		
Homarus gammarus	microcapsules, microparticulate	no survival to post larva Kurmaly et al. (1990).

and feed at an early stage (2-3mm) and therefore success with total feed replacement for marine larvae is extremely limited.

Langdon et al., (1985) reviewed extensively different processing techniques required to produce artificial particles in dehydrated forms as food for aquatic animals. Artificial diets must satisfy the following criteria: acceptability, digestibility, stability, adequate nutritional content, cost effectiveness and storage (Jones et al., 1993). Jones et al., (1972) discovered that some filter feeding crustaceans accept artificial food particles. Since then several kinds of artificial diets have been manufactured to replace live feeds partially or totally. The most commonly used artificial diets to culture shrimp are microbound (microparticulated) and microencapsulated diets (MED). Microbound diets are inexpensive and easy to produce and although these leach rapidly are reported to be used successfully in laboratory and hatcheries (Kanazawa, 1990).

Table4b. Summary of the live feed replacement for first feeding fish larvae. After Jones et al. (1993).

Species	Feed replacement	Result
Cyprinus carpio	microparticulate diet	90% survival, growth comparable with live feeds Charlon and Bergot (1984)
Coregonus lavaretus	microparticulate (yeast based)	85-95% survival mass rearing, good growth Champigneuille (1988)
Micropterus dolomieni	commercial dry diet	26-45% survival growth better than Artemia control Herlich et al. (1989).
Morone saxatilis	microparticulate	no survival at 20 d Tuncer et al. (1990)
Plecoglossus altivelus	zein microbound	good growth and survival Kanazawa et al. (1985).
Marine		
Pleuronectes platessa	microparticulate	survival 50% of control on live feeds Adron et al. (1974)
Solea solea	Microparticulate/ microencapsulated	lower survival and growth than live fed controls Appelbaum (1985)
Solea solea	zein coated particles	lower survival and growth than live fed controls Gatesoupe et al. (1977)
Dicentrachus labrax	zein coated particles	lower survival and growth than live fed controls Gatesoupe et al. (1977)
Sparus aurata	micro diet with/without exogenous enzymes	best survival and growth with enzymes still less than live feeds Kolkovsky et al. (1990).
Lates calcarifer	microcapsules	no survival after 10 d Walford and Lam (1991).
Gadus morhua	microencapsulated cod roe	poor growth and survival Garatun - Tjeldsoto et al. (1989)
Clupea harrengus	encapsulated cod roe	poor growth and survival Fox (1990).
Pagrus major	microcapsules, zein coated, microbound	little survival and growth Kanazawa et al (1982).

Diets are produced by mixing the nutritional ingredients thoroughly with binders; carboxymethyl, cellulose, calcium alginate, carrageen, agar and gelatine. The mixture is then freeze dried, ground and finally sieved through appropriate sizes. They exhibit poor stability in water causing not only water pollution and bacterial build up, but also they may become nutrient deficient due to the leaching process in water (Amjad et al, 1992).

Complete replacement of live diets with MED has had limited success, partial replacement is already routinely used in many hatcheries (Jones et al.,1987; Fegan, 1992). In general penaeid

larval growth and development on live feeds is generally superior to those reared on formulated diets (Galgani & Aquacop, 1988; Jones et al., 1993). Complete replacement of live diets by artificial diets to rear caridean shrimp and homarid larvae is not currrently possible. Live *Artemia* was replaced completely in *Macrobrachium rosenbergii*, but only from stage Z6 to Z11 with microencapsulated diets designed for penaeid larvae (Deru, 1990). The production of *M. rosenbergii* still relies on *Artemia* at least during it's early stage of development (Jones et al., 1993).

The inability to survive on artificial diets may be due to their feeding behaviour and low digestive enzyme activities (Jones et al., 1993). If a total replacement feed for algae and *Artemia* becomes routine for penaeid culture, rearing costs will be greatly reduced (Ottogalli, 1991). However currently live food organisms are considered to be superior in terms of survival and growth to other feeds. Possible reasons are that living organisms are ingested more easily and have essential factors such as enzymes that are absent or lost in a non living feed (Munilla-Moran et al., 1990).

LARVAL DIGESTION AND NUTRITION.

Penaeid shrimp obtain their food by filtering micro algae from water at the protozoeal stages, and capturing zooplankton at mysis and postlarval stages. Caridean larvae however consume zooplankton directly 24 - 36h after hatching. The size of micro algae used to feed early shrimp larvae ranges from 5 - 20µm in diameter, whereas the size range of animal prey ranges 70 - 500µm. It is thought that *Artemia salina* nauplii are the only realistic live prey for both penaeid and caridean larvae such as *M. rosenbergii* until their early postlarval stages (Kumlu and Jones, 1995). Although there is much research in the nutritional requirement of adults and juveniles, little is known about the feeding mechanism, digestion, digestive enzymes assimilation, gut structure and nutritional requirement of larval decapods.

It can be concluded that penaeid larvae require a protein level between 23 - 55% of dry weight of the diet (Liao and Liu., 1990; Akiyama et al., 1992; Rodiguez et al., 1994). Jones et al.,

(1979) showed the importance of HUFA particularly 20:5ω3 and 22:6ω3 in *Penaeus japonicus* using nutritionally defined microcapsules. Lipid and carbohydrate levels used in penaeid culture contain 52% protein, 13-14% carbohydrate, 12% lipid, 2% HUFA (Le Vay, 1994). Essential vitamins are generally included in artificial diets at levels higher than recommended (Kanazawa, 1990).

An extensive review on the nutritional research on penaeids was undertaken by New (1976, 1980). Despite recent advances in understanding of adult and juvenile decapod crustaceans (Kanazawa, 1984, 1990; Lia and Liu, 1990; Guilame, 1990; Chen, 1993). Only limited information on specific dietary requirements for crustacean larvae is currently available (Jones et al., 1979a,b., Kurmaly et al, 1989b; Jones et al., 1993). The absolute nutritional requirements of penaeid shrimp can only be identified when a wet stable formulated diet is accepted digested and assimilated comparable to live diets (Jones et al., 1993).

USE OF NEMATODES FOR FISH FEED.

The use of the nematode *Anguilla aceti* met with great success in Denmark in the 1930s for keepers of tropical fish. Larsen (1941) gave an historical account of the use of nematodes as a fish feed. The following species of fish could be raised on nematodes; *Aphyocypris*, *Barbus, Betta, Cichlasoma, Colisa, Cyprinodon, Dermogenys, Hemichromis, Hemigrammus, Hyphcisobrycon, Melantaenia, Namhoshomus, Trichgaster* and others (Bruun, 1941). Examination of the stomach contents of fish demonstrated that nematodes are eaten by various fish larvae (Thomson, 1963, 1966; Vallet et al., 1970). Hofsten (1983) fed free living species of nematodes *P. redivivus*, *Turbatrix aceti, Caenorhabditis elegans* and *C. briggsae* to *Danio sps* (Table 5). After 3 h of feeding nematodes, none were observed in the guts of the fish except for the buccal capsules of the nematodes and males' spicules. This indicated that nematodes are probably preyed upon by fish.

Rottman et al., (1991) explored the possibility of using nematodes for feeding to grass carp (*Ctenopharynyodon idella*) and bighead carp (*Hypthalamichys nobilis*). In the wild, rotifers are the initial live food for grass and bighead carp followed by cladocerans and copepods

Table 5 Percentage of various nematodes digested and sites of disintegration in the nematode body after duration of 0.5-1h in the alimentary canal of the fish *Danio sps* (average +/- S.D.). After Hofsten, (1993).

	Percentage of digested nematodes	Digested at extremities only (anterior and /or posterior	Digested at middle of body only	Evenly digested along whole length of body	Other*
Panagrellus sps	73.0+/-18.2	80.4+/-5.9	12.8+/-6.8	0.2+/-0.5	5.8+/-3.4
Turbatrix aceti	48.7+/-12.7	62.5+/-13.3	2.0+/-2.2	5.5+/-7.5	30.0+/-14.9
Caenorhabditis elegans	54.0+/-19.3	12.5+/-12.9	0.5+/-0.5	57.0+/-18.7	30.5+/-12.1
Caenorhabditis briggsae	41.3+/-11.1	31.4+/-10.3	3.5+/-2.5	25.7+/-13.8	39.4+/-9.3

*Fragmented bodies and advanced stages of digestion with cuticle intact.

(Dabrowski, 1984). It was suggested that larvae should be raised on rotifers initially followed by *Artemia* (Van der Win, 1979). A three week feeding trial showed that larvae fed fresh water rotifers (*Brachionus rubens*) were consistently longer and heavier at the end of this period than those that were fed on nematodes (*Panagrellus sps.*) (Table 6 and 7). The length of the bighead carp fed *Panagrellus* nematodes were not significantly different from those fed *Artemia*. Growth of grasscarp fed two commercial dry diets was less than those fed the three live feeds. The minimum feeding for fish fed nematodes was not explored although this could be an important factor in the success of fish feeding trials as it would be cost effective. The results proved promising from the point of view of using freshwater rotifers however they do not survive for long periods of time. As a result, intensive culture techniques for freshwater rotifers have not received as much attention as the marine rotifer *B. plicatilis*. In contrast the nematode *Panagrellus sp.* as discussed earlier is easy to culture (Rottman, 1988: Ivleva. 1969).

Further to the above study Kahan et al., (1983) explored the use of nematodes as a dietary supplement for rearing fish fry in hatcheries. The objective of this study was to observe the effects of growth of newly hatched fry of common carp *Cyprinus carpio* and silver carp *H. molitrix* by feeding nematodes and dry feeds. Feeding nematodes to the fish larvae without the addition of dry feeds gave satisfactory results, however when nematodes were fed in conjunction with dry feeds, the growth was 1.39 to 1.84 times better with higher survival than diets without the addition of nematodes.

Table 6. Growth and survival of grass carp fed different foods. After Rottman et al.,(1991).

Diet	Week 1		Week 2		Week 3		
	Length (mm)	Weight (mg)	Length (mm)	Weight (mg)	Length (mm)	Weight (mg)	Survival %
Artemia	10.1+/-0.1	34.9+/-3.9	10.9+/-0.2	87.9+/-6.4	16.0+/-0.3	308.9+/-38.6	87.0+/- 11.1
B. rubens	8.7 +/-0.1	20.9+/-0.8	12.5 +/-0.2	120.5 +/- 12.3	18.4 +/- 0.3	503.1+/- 45.7	77.2+/-4.9
Panagrellus	8.5 +/-0.1	29.9+/-0.3	11.7+/-0.2	103.3+/-6.6	15.6+/-0.2	239.4+/-12.0	95.1+/-0.8
Ewos	8.4 +/- 0.1	23.0 +/-2.5	9.5 +/-0.1	46.9+/-0.3	12.3 +/-0.2	149.0+/-27.4	89.6+/-2.9
Kyowa	8.1+/-0.1	25.8+/-0.8	9.4+/-0.1	54.6+/-4.0	11.5+/-0.2	114.5 +/-8.1	63.3+/-10.7

Table 7. Growth and survival of bighead carp larvae fed different foods. After Rottman et al.,(1991).

Diet	Week1		Week2		Week3		
	Length (mm)	Weight (mg)	Length (mm)	Weight (mg)	Length (mm)	Weight (mg)	Survival %
Artemia	11.8+/-0.1	48.8+/-0.3	14.7+/-0.1	165.9+/-5.0	16.0+/-0.2	226.2+/-22.7	98.8+/-1.0
B.rubens	14.1+/-0.1	98.6+/-0.2	17.9+/-0.2	317.0+/-2.6	19.8+/-0.3	402.9+/-0.4	99.3+/-1.8
Panagrellus	11.7+/-0.1	49.9+/-0.9	14.0+/-0.1	137.2+/-5.1	16.4+/-0.1	196.1+/-4.1	96.1+/-0.7
Ewos	11.7+/-0.1	48.5+/-1.2	13.6+/-0.2	141.1+/-9.8	14.8+/-0.3	211.6+/-11.5	89.7+/-1.3
Kyowa	11.7+/-0.1	69.1+/-5.3	14.5+/-0.2	217.8+/-14.1	16.7+/-0.2	351.7+/-35.8	82.5+/-0.5

The resistance of some parts of the nematodes to digestion is due to the structure of the cuticle (Hyman, 1951; Lee, 1966; Bird, 1971). Nematodes are digested in the fish intestine (Kahan and Appel, 1975; Kahan, 1979 and Kahan et al., 1980). Differences were observed in the digestion of various nematode species. For *Panagrellus* and *Turbatrix*, digestion mainly occurred at the anterior and posterior extremities when observed in the fish gut. In *Caenorhabditis sp.* the digestion was initiated at the nematode's natural body apertures, mouth anus and cloaca (Hofsten, 1983). The cuticle is composed of a cortical and basal layer. The most resistant layer is the external cortex and striated structure of the basal layer which consists of proteins with a very close intermolecular linkages due to the disulphide and sulphurhydryl groups, polyphenols and quinone bonds in addition to a thin superficial lipid layer. This striated layer is absent from *Panagrellus* and *Turbatrix sps.*

The use of live nematodes together with dry feeds may provide a solution to problems encountered elsewhere in the rearing of silver carp (Opuszynsk, 1977; Rychly, 1977). It was also previously stated that the nutritional value of the nematodes could be improved by loading the alimentary canal with growth factors for fish (Lorentz, 1960).

The value of *Panagrellus sp.* as food for fish was investigated in trials using *Danio malbricus* and *Poecilia reticulata* (Kahan and Appel, 1975). The survival of *D. malabaricus* larvae fed nematodes was higher than when compared to the control diet. Growth of *P. reticulata* larvae fed nematodes was little better than compared to approximately 23mg for control. Experiments were conducted to try to adapt *Panagrellus sp.* to sea water, this showed that nematodes can grow at a low rate at 30% salinity. Addition of diluted seawater to the medium improved the growth of the nematodes. Nematodes of this genus are known to survive drastic environmental changes and are only damaged after prolonged exposure to extreme conditions (Ivleva, 1969). Free living nematodes have higher growth rates compared to marine nematodes (Tietjen and Lee, 1973).

Kahan (1984) emphasised that it was important to evaluate other food sources such as nematodes and copepods because of their 1) small size 2) fast growth 3) tolerance of seawater 4) nutritive value for aquarium (*Danio malabaricus*) and commercial fish fry (*Cyprinus carpio, Hypthalmichthys molitrix, Mugil capito, Sparus aurata*) (Kahan 1979, 1982; Kahan et al, 1980). These fish can digest the hard cuticle of various nematodes (Hofsten et a.l, 1983). Crustaceans such as penaeids and *Macrobrachium rosenbergii* seem to ingest harpaticoid copepods and these are routinely used in marine hatcheries in Japan (Kuronuma and Fukusho, 1984).

NEMATODE PHYSIOLOGY AND BIOCHEMISTRY.

There are over 60,000 known nematode species and all are similar in shape, having an elongated bilateral body tapering at both ends. There is no segmentation or appendages and there is very little variation in shape. An identified nematode can be placed in one of two subclasses; Adenphorea or Secernetea. Apart from the normal method of classifying nematodes on morphological characteristics, Hansen and Buecher (1970) have described a biochemical approach. Biosynthetic pathways are of great significance, for example the ability to synthesise

certain polyunsaturated fatty acids which has been lost by higher animals, is present in the axenic culture of *Turbatrix aceti*. The advent of the production of axenic nematode has made it possible to obtain more accurate classification using these methods. Sensitive lipid analysis has shown genetically based differences in a strain of *Panagrellus redivivus* selected for tolerance to an increased temperature of 32^0C. The lipid composition was very different from that of xenically cultured species (Hansen and Buecher, 1970).

DIGESTION AND ABSORPTION

Nematodes feed on a wide variety of substrates including bacteria, fungi, algae, protozoa, other nematodes and soil microfauna. Feeding is achieved by the production of a variety of digestive enzymes. Absorption occurs by way of the intestine and not through the cuticle (Rogers and Lazarus, 1949). The nematode intestine contains an abundance of acid and alkali phosphatases thought to be essential for the absorption of simple sugars. Nematode tissues contain little glucose and considerable quantities of the disaccharide trehalose.

All nematode tissues are bathed in haemolymph which fills the body cavity and is always under pressure. The majority of physiological and biochemical research has been undertaken on *Ascaris sp.*as this is thought to be the most representative of the groups of nematodes and it is on this research that the majority of nematode functions are based. *Ascaris* haemolymph contains proteins, carbohydrates and fats. Proteins are 4.9% of the fresh weight and 66% of the total solids, these consist primarily of albumin and globulins. Haemoglobin is present in small amounts (Davenport, 1949). Several enzymes have also been identified; invertase, maltase, amylase and protease (Cavier, 1951).

Carbohydrates are abundant in the haemolymph consisting of 0.4% glycogen and 0.77% trehalose. Glucose and other carbohydrates occur in traces. Fats are less abundant comprising of significant amounts of phospholipids (0.18%) and triglycerides (0.13%) (Cavier et al., 1958). Important differences and similarities occur amongst species of nematode so that their ionic composition might be expected to reflect the conditions under which a particular species lives and may change from development stage to another.

ASSIMILATION AND CHEMICAL COMPOSITION.

In all nematodes the amounts of energy required for motion are probably very small but in comparison the needs of the female reproductive organs are impressively high. Total glucose ranges widely in species from 0.01% of their dry weight in filarial nematodes to 0.78% in *Ascaridia galli*. Trehalose ranges from 2.18% in the codworm larvae *Porrocaecum decipiens* to 0.06% in *L. carnii*.

LIPIDS.

The first detailed study of the lipids of nematodes was undertaken by Flury (1912). He showed that the lipids represented were those of higher animals but there were important differences. Reproductive organs are the most important sites for lipid deposition in *Ascaris* (Fairbairn, 1955). Lipids generally comprise 17.5% of the dry weight. The composition of the triglyceride and phospholipid fraction is not dissimilar to that of higher animals. There is a great deal of evidence that nematodes undertake the synthesis of lipids some of which are incorporated in the somatic tissues but the majority is required by the female for the production of eggs. Triglycerides serve as an energy source.

PROTEINS.

Proteins are the structural components of cells and tissues. There is little evidence in nematodes that protein is stored for use in energy metabolism during prolonged starvation. Large amounts of protein are deposited in the oocytes of *Ascaris* where large amounts of albumin and globulins are present. Two percent of the total protein is contained in haemoglobin, no species of nematode has been shown to lack this protein. However this protein does not have an oxygen carrying capacity in the nematode as it has very high affinity for oxygen. Even at low partial pressures of this gas there is no dissociation of oxyhaemoglobin except at very low tissue concentration of oxygen. A quantitative study of this phenomenon was made by (Roger and Lazarus, 1949;Davenport, 1949) and it was concluded that oxygen transportation in *Ascaris* and *Nippostrongyloides* is improbable.

The cuticle is produced from secretions from the underlying epidermis, 75% of which consists of water and the rest consists of protein. The basement membrane is secreted near the epidermis, then three fibre layers which consists of collagen although many species only have two layers. Little research has been carried out on the juvenile membrane.

Most nitrogen metabolism in the adult female is directed towards the extensive synthesis of ovarian proteins which are deposited in the oocyte as yolk material to be incorporated subsequently into the structural protein of the embryo. During embryonation the yolk is converted to the many different proteins necessary for various cells and tissues (Passey and Fairbairn, 1957). Spermatogonia of many nematodes (Chitwood and Chitwood, 1950) contains granules which fuse to form the prominent acrosome of the sperm protein called ascardine. This has been isolated from *Parascaris* sperm (Filhol, 1937) and was found to contain 2 amino acids aspartic and trytophan which are approximately 35% and 15% of total nitrogen content respectively.

PRESENT RESEARCH AIMS

Nematodes are proposed as a possible live food replacement in penaeids however the limiting factor is the mass culturing of these organisms. Vast quantities are required for the later substages of penaeid larviculture to obtain a growth equal to that of *Artemia* in commercial hatcheries. Hence one objective of present research is to address this problem. The aims of chapter 1 are to select a suitable nematode species and optimise it's culture. This is undertaken by exploring the various constituents of the media to be used to provide an adequate nitrogen, carbohydrate and lipid source. Optimum inoculum level and temperature are to be explored for the selected species of nematodes that produce optimum levels in culture. Further aims are to culture these nematode species in 10 litre fermenters which provide similar conditions to industrial scale 1000 and 10,000 litre fermenters.

As the literature review shows live feeds such as *Artemia* may be nutritionally inadequate and dry feeds nutritionally unstable. Research in chapter 2 aims to optimise lipid enrichment of the chosen species of nematode, by incorporating into the optimised medium fish oils that are high in Highly Unsaturated Fatty Acids (HUFA). Such as eicosapentaenoic acid 20:5ω3 and

docosahexaenoic acid 22:6ω3. Preliminary trials at Bangor University with penaeid larvae (chapter 5) indicate that larvae survived and grew well on nematodes compared to *Artemia* fed controls, but these larvae lacked the pigmentation observed with larvae feeding on the *Artemia*. The objective of chapter 3 is to attempt to pigment the nematodes via the medium using industrially produced pigments such as astaxanthin and castaxanthin. This would enable the nematodes to become more visible to both fish and shrimp larvae and assist in the pigmentation of the penaeid larvae.

If nematodes are to be any use to the aquaculturists on a commercial scale then successful nematode species that can be cultured on a large scale and lipid enriched will require storage for long periods of time at local ambient temperatures and must be capable of surviving. Chapter 4 explores the possibilites of storage of nematodes in a variety of inert carriers which provides adequate protection from dessication and nutritional degradation during storage. In chapter 5 the above products are tested with suitable penaeid shrimp and fish larvae. The shrimp trials were undertaken in collaboration with Dr. David Fletcher at Bangor University. Fish larval trials were undertaken at the Hunterston Fish Laboratories in Argyll by Andrew Barbour.

CHAPTER ONE

CULTURE OF BACTERIAL FEEDING NEMATODES

1. CULTURE OF BACTERIAL FEEDING NEMATODES.

INTRODUCTION

Nematodes have been suggested as possible live food replacement for penaeids and some fish larvae (Plagman, 1939; Nikolsky,1963; Thomson, 1963, 1966; Gerlach and Schrage, 1969; Vallet et al, 1970; Opuszynsk, 1977; Rychly, 1977; Kahan et al,1975, 1979, 1980, 1983, 1984; Hofsten et al, 1983; Wilkenfeld et al, 1984; Biedenbach, 1989; Rottman et al, 1991). However the limiting factor is the mass culturing of these organisms. Vast quantities are required for the later substages of penaeid larviculture to obtain a growth equal to that of *Artemia* in commercial hatcheries.

A common method of raising nematodes is by placing them on nutrient agar plates with their associated bacteria. The associated bacteria are streaked onto the agar plates and incubated 24h before the addition of the nematodes. This method also allows for observation under the microscope. To establish bacterial feeding for soil nematodes soil humus can be added to the agar. Species of Acrobeloides and the majority of Rhabditidae can be cultured in this way (Nicholas et al., 1959). However for the commercial production of nematodes such *Steinernema feltiae*, *Phasmarhabditis hermaphrodita* and *Heterorhabdtis megidis,* which are used extensively in the horticultural market, nematodes are cultured monoxenically in 1000 litre fermenters.

The objective of this chapter is to select nematode species for monoxenisation and to optimise their culture on a small scale using current fermenter technology. The aim is to produce quantities of nematodes that would be comparable to that of *Artemia* on an industrial scale.

Monoxenic versus Axenic cuture.

Axenic cultures consist of only one known organism and monoxenic cultures include one other associated organism. The advantages of the production of nematodes on an industrial scale in a monoxenic culture far exceed those of axenic culture. The production of the entomopathogenic nematodes require the presence of the symbiotic bacterium *Xenorhabdus sp* before infection of

a host will take place. However the cost of producing nematodes by insect infection is high, estimates of cost exceed 1 dollar U.S. per million infective juveniles. The cost of raw materials for an axeinc culture are high and require complex components. Growing nematodes with their associated bacterium requires a medium that is inexpensive. It was observed that the symbiotic bacterium was necessary for the success of these cultures (Dutky et al, 1967). The symbiotic bacterium *Xenorhabdus sp.* provides significant nutritional benefits in a production system and allows the use of an inexpensive medium materials on a large scale.

Tables 1 and 2 summarises the axenic and monoxenic culture of nematodes. The objective of this literature review was to become familiar with the materials and methods used to culture nematodes and to apply these techniques for species that have not been cultured in liquid before. From this review various medium components were selected and tested with the nematode species.

To obtain optimum growth in a monoxenic culture the following parameters were investigated for the selected nematode species; **inoculum level**, **temperature optimisation** and **medium components.** *S. feltiae* and *H. megidis* require an initial inoculum level of 2000 nematodes per ml and 1800 nematodes per ml respectively. If these numbers are reduced or increased the overall yield is reduced along with the mixture of growth stages in the final culture (Pearce, pers. comm.). Few authors have investigated the effects of the initial inoculum level. Gbewonyo et al, (1994) describes using 'worms' from 50 agar plates as inoculum for 4 litres of liquid medium for the large scale cultivation of *C. elegans.* An initial inoculum level has a profound effect on the overall yield. The following experiments describe the investigation of the initial inoculum level for two free living species.

Two experiments were undertaken to investigate the initial inoculum level of *Panagrellus redivivus* and *Caenorhabditis elegans.* Further experiments were then conducted to investigate the optimum temperature of growth for these species of nematodes.

It has been established from previous work that a variety of nematode species including *P. redivivus* can be successfully grown and subcultured in a liquid medium containing 10% kidney,

3.5% corn oil and 1% yeast extract (AGC, MicroBio Ltd.). This is a rich and expensive medium

Table 1. Summary of Axenic culture of nematode species

AXENIC CULTURE		
NEMATODE SPECIES	MEDIUM	AUTHORS
Caenorhabditis elegans, C. briggsae, Rhabditis anomala, R. maupasi, R. marina, Pelodera strongyloides, Acrobeloides buetschlii, Chiloplacus lentus, Turbatrix aceti, Aphelenchus avenae, A. rutgersi, Neoaplectana	general axenic culture review	Vanfleteren, (1978).
C. lentus	YE, glucose and supplement S7 (24 amino acids, distilled water and HCL	Roy,(1975).
C. elegans	YE, soy protein, dextrose,vitamins and HLE	Buecher and Hansen, (1970).
Aphelenchoides spp	basal medium supplemented with trehalose	Myers, 1968; Petriello and Myers, (1971).
A. avenae	fresh chick embryo and serum	Hansen and Buecher, (1970).
P.strongyloides	soy peptone, YE and HLE	Yarwood and Hansen, (1968).
P. strongyloides	liver peptone, YE, 250µm of growth factor and 8.2% Ficoll : liver protein, YE and 20% HLE	Buecher et al, (1966).
P. redivivus, T. aceti	activated protein growth factor in a medium containing soya peptone, YE and HLE. For T. aceti 5% of 95% ethanol added	Hansen and Cryan (1966).
C. briggsae, P. redivivus	soya and peptone	Cryan, (1963).
T. aceti, P. redivivus, R..anomala, C. elegans	basal medium(Sayre et al, 1963), but amino acids replaced by soya peptone	Rothstein and Cook, (1966).
Trichinella spiralis	plasma-chick extract medium	Berntzen, (1965)

Table 1 contd.

Acrobeloides buetschlii	unidentified vitamin, chick embryo extract, liver protein and HLE	Nicholas, (1962).
Neoaplectana glaseri	raw liver and veal infusion broth	Stoll, (1953).
N. glaseri	agar slants with sterile rabbit kidney	Glaser, (1940).
T. aceti	peptone glucose, YE, Na CL and lecithin	Zimmerman, (1921)

Table 3. Summary of the Monoxenic culture of nematode species.

MONOXENIC CULTURE		
NEMATODE SPECIES	MEDIA	AUTHORS
Caenorhabditis elegans	nutrient agar slants	Grewal, (1989).
Steinernema feltiae, Heterorhabditis megidis, Phasmarhabditis hermaphrodita	media components confidential	MicroBio Ltd, (1995).
S. feltiae	egg yolk, vitamins, minerals and a triglyceride.	Friedman, (1989).
S. feltiae	ox kidney, YE	Pace et al, (1986).
N. carpocapsae, H. megidis	polyether polyurethane sponge coated with kidney/beef homogenate	Bedding (1984)
N. carpocapsae	dog food agar medium	U.S. Dept. of Agriculture. (1981).
S. feltiae	polyether polyurethane sponge coated with potato broth	Wouts et al, (1981).
S. feltiae	soya peptone. YE and dextrose	Buecher et al, (1975).
S. feltiae	agar, nutrient broth,YE, soya flour and corn oil	Dutky et al, (1967)
Rhabdititidae, Diplogasteridae, Panagrolaimidae, Cephalobidae	$MgSO_4.7H_20$ (0.75g), K_2HPO_4 (0.75g), NaCl (2.75g), KNO_3 (3g), peptone (2.5g), lecithin (1g), agar (15g), distilled water 1 litre	Nigon (1949)

and therefore it is necessary to investigate alternative media that are cheaper and more convenient to use on a larger scale. Experiment 5 in this chapter investigate the effect of different liquid media on the growth and development of the nematode *P. redivivus*. The 5 types of media used on a commercial basis in fermenters for the commercial production of entomopathogenic nematodes at MicroBio Ltd. were investigated. The percentage of media components are specific to the nematode grown, therefore it was necessary to investigate further individual components to optimise the growth media for *P. redivivus*. At the time of this experiment new media components were being investigated at the laboratories of of AGC MicroBio Ltd. which were far cheaper and were giving culture numbers comparable to the original commercial media. Experiment 5 describes the new media components which were evaluated. It was found that a media containing bovril showed very promising results and it was simple to use and cheap in comparison to live tissue. The component percentages of bovril to egg were then investigated to obtain the optimum percentages in the medium for growing *P. redivivus*. Following on from the results obtained from the above experiment higher percentage of solids in the medium were investigated to observe if this increased the numbers of nematodes obtained in the culture medium. It was found that the concentration of yeast extract in the medium affected the overall population numbers of the nematodes Finally the optimum concentration of yeast extract in the medium was evaluated as the combination of egg and yeast extract proved detrimental to the growth of the nematodes at some concentrations.

Experiment 12 describes the use of the optimised medium obtained from the above results containing 2% bovril, 1.5% egg and 1% yeast extract and cultured the nematodes in a 10L fermenter. The results from the experiments indicates the possibility of growth in larger vessels.

MATERIALS AND METHODS

Sterilizing nematodes

Free living species of nematodes were obtained from a variety of sources; *Caenorhabditis elegans, C. remanei, C. vulgaris* were obtained from Ann Burnell (Saint Patricks College Maynooth, Ireland). *C. briggsae* (Caenorhabditis Genetics Centre, Missouri). *Panagrellus redivivus* Professor John Barrett (Aberyswth University). *Panagrolaimus sps.*, *Rhabditis pellio* (Rothamstead Experimental Station).

Species of *Caenorhabditis elegans, C. briggsae, C. remanei, C. vulgaris* were received on agar plates growing monoxenically with *Escherichia coli.* However plates were damaged during transit and were not monoxenic when they arrived. *Panagrellus redivivus* was received and maintained on a xenic culture medium of autoclaved oats described by Barret and Butterworth, (1984). *Rhabditis pellio* was received growing on Nigon's media as described earlier.

Insect parasitic nematodes such as *S. feltiae* and *H. megidis* were obtained from John Godliman (MicroBio LtD). *Phasmarhabditis hermaphrodita* was obtained from Mike Wilson (Long Ashton Research Station). *S. carpocapsae* and *S. anomali* were obtained from Roma Gwynn (Horticulture Research International Littlehampton).

Before antibiotics were considered, chemicals such as hydrogen peroxide were used to sterilise nematodes (Zimmerman, 1921). Briggs (1946) used a combination of hypochlorite and controlled doses of U.V. light to axenize eggs of *C. briggsae.* Nematode species from Tylenchida are easier to sterilise because their stylets are too narrow to allow the passage of bacteria into their guts and therefore bacteria are not present in the gut as they are in Rhabditida.

Nematodes that are to be cultured in an axenic or monoxenic environment need to free of contaminating bacterial flora. For an axenic culture this means removing all known bacteria associated with the nematode.

Monoxenisation of free living nematodes species.

For monoxensation of *P. redivivus and C. elegans,* nutrient agar plates were inoculated with *Escherichia coli* and incubated for 24 hours. The nematodes were added to the plates and incubated for 3 - 4 days or until gravid females were observed. A technique described by Wilson and Glen (1990) was used to axenise the nematodes. Approximately 20 gravid females were removed from agar plates and placed into a watch glass containing sterile distilled water. This was done so that any remaining debris or agar was washed off, twelve repetitions were made. The following procedure was undertaken under sterile conditions; the gravid females were then placed into watch glasses containing merthiolate (ethyl mercurithiosalicylate). Four concentrations were tested 1) 0.02% (Wilson and Glen, 1990) 2) 0.015% 3) 0.01% 4) 0.005% (w/v), 3 repetitions for each treatment were used. The watch glasses were placed in covered petri dishes and incubated at 17^0C for 12 h, this encouraged the hatching of the juveniles. After 12 h the juveniles were removed using micro pipettes and placed into 15ml centrifuge tubes containing quarter strength Ringer's solution containing 500 units of streptomycin sulphate and Penicillin G. The tubes were incubated at 5^0C for a further 24 h. After 24 h the nematodes were concentrated by centrifugation at 200G for ten minutes. The supernatant fluid was then removed by pipette and the juveniles were suspended in quarter strength Ringer's solution to remove any remaining mercury ions, this process was repeated twice.

Nematodes were removed from the tubes using micro pipettes and placed onto the centre of nutrient agar plates which had been inoculated on one side of the plate 24 hours previously with *Escherichia coli* to test for contaminants remaining with the nematodes (these were spotted within 24 h incubated at 24^0C). Nematodes were then maintained on agar plates and regularly reinoculated into fresh plates.

Medium used successfully for Rhabditidae, Diplogasteridae, Panagrolaimidae and Cephalobidae nematodes, was prepared with the following components;

$MgSO_4.7H_2O$ 0.75g

K_2HPO_4 0.75g

NaCl 2.75g

KNO_3 3.00g

peptone

(bacteriological) 2.50g

lecithin 1.00g

agar 15.00

distilled water 1 litre

Lecithin was dissolved in 25ml of absolute alcohol. Salts were dissolved in one litre of distilled water and then peptone and dissolved lecithin. Powdered agar was added and autoclaved at 15lb ($1.05kg/cm^2$) (121^0C) for 15min. The mixture was then standardised at pH 7.2 by the K_2HPO_4. Medium was poured into sterile dishes. Many rhabditids will establish themselves on this medium and support the growth of associated bacterium.

Culture and handling of *C. elegans* using nutrient agar plates with *E. coli* strain OP50 have been described by Brenner (1974).

C. elegans and *P. redivivus* stock was grown at 20^0C on *E. coli* strain OP50 in 9cm petri dishes containing agar. Cultures were maintained by transferral of $1cm^2$ blocks of agar from plates containing mature nematodes, using a flame sterilized scalpel, onto agar plates freshly streaked with *E. coli.* Cultures were maintained on nutrient agar slants at 4^0C.

Phasmarhabditis hermaphrodita is most commonly found parasitising slugs. It can be monoxenised by the same technique as described above. *E.coli* is replaced by the bacterium *Moraxella phenylpyruvica.*

Monoxenisation of insect nematodes.

The insect parasitic nematodes such as *Steinernema feltiae, S. anomali, S. carpocapsae* and *Heterorhabdidis megidis* can be obtained in high numbers by infecting *Galleria mellonella* larvae. Individual larvae of *G. mellonella* were placed onto damp circles of filter paper in petri dishes and incubated at the appropriate temperature for each species, 18^0C for *Heterorhabditis sp* and 22^0C for *Steinernema spp.* After 2 weeks of incubation the infective stage juveniles can

be obtained from the dead larvae.

Pure strains of the associated bacterium of the nematodes can also be obtained from the infected larvae. Infected larvae were removed from the plates as soon as the larvae were dead and before the carcass erupts releasing the infective stage larvae. The larvae were then dipped in alcohol and flamed to remove bacteria on the surface of the larvae. Using sterile forceps the cuticle was split to allow a bubble of haemolymph to form. A sterile loop was dipped into this bubble and plated onto agar plates containing TTA (Tetrazolium salt). The plates were incubated at 18^0C, after 48 hours bacterial colonies can be detected. Bacterium belonging to the genus *Xenorhabdus* is found associated with Steinernematids and Heterorhabditids and can be detected by the fact that the colonies will take up the pigment from the TTA agar plates if the bacterium is present in it's primary form. In their secondary form these bacterial colonies are pink. The primary form of this bacterium is known to be associated with the infective stage juveniles.

For surface sterilising *Steinernema* species 8 treatment repetitions were used for each isolate, in two sets of 4. Starting the 4 at three min intervals. This allows time for all procedures as they take 10 min in NaOCl. *Heterorhabditis spp* can only take 4 minutes. There also seems to be variation with some *Steinernema sp* isolates.

The method required the use of: sterile embryo dish, 30ml universal bottle containing 20ml sterile tap water; 2ml 10% NaOCl (10% of stock); 3 sterile pipette tips to take 0.2ml; slope diet tube.

1) Several hundred nematodes are placed into 2ml of NaOCl in the embryo dish and swirled. Dead nematodes were avoided as they harbour contaminants.

2) After 10 min for *Steinernema* and 3 min for *Heterorhabditis*, the dish was swirled gently to centralise the nematodes and picked up in 0.2ml.

3) Nematodes were then placed in sterile tap water (flaming the neck) and the lid was replaced loosely.

4) Bottles were sloped for about 15 min.

5) The lid was then removed and flamed, 0.2ml is pipetted from the bottom corner of the bottle, sweeping from side to side. This collects the nematodes.

6) Nematodes are then added to a sterile slope(kidney agar slope), flaming the neck. The slope had been inoculated with pure *Xenorhabdus* of the same isolate 24 h previously

7) Slopes were then placed in an incubator at 18^0C .

Culturing insect parasitic and free living nematodes

1) Kidney agar slopes were left for 2 -3 weeks until infective nematodes could be seen migrating onto the glass of the tube. Agar plates containing free living species were left up to 7 days or until nematodes were proliferate.

2) Nematodes were then transferred under sterile conditions into 50ml flasks containing 15ml of kidney flasks medium inoculated 24 h previously with bacterium. Flasks were left for 2-3 weeks in a shaking incubator at 15^0C for *P.hermaphrodita* and 22^0C for all other species of nematodes until infective nematodes were seen in the medium or before the medium was spent.

3) Further nematode cultures can be started by transferring known numbers of nematodes into fresh flask or sponge diet under sterile conditions.

4) Nematode population assessments were sampled under sterile conditions. 1ml aliquots were taken from the medium and diluted in either 10 ml, 50 ml, 100 ml, 500 ml or 1000 ml depending on the concentration of nematodes. 4 replicate counts were taken for each sample.

Kidney agar slope diet:

10% kidney

1% yeast extract

3.5% corn oil

1.2% agar technical

84.3% water

Approximately 6ml per universal tube. Autoclaved and shaken to disperse lumps then sloped to set.

Kidney agar nematode flask diet.

10% kidney (wet weight)

3.5% corn oil

1% yeast extract.

Ingredients were homogenised and 50 ml aliquots are placed into 250ml baffled flasks and autoclaved.

Extraction from flasks

1) Flasks were filled with sterile water and placed on a slant in a flow cupboard. After about 2 h or until the nematodes had settled the supernatant was decanted. The process was repeated until the supernatant was clear.

Experiment 1

Optimisation of inoculum for *P. redivivus*

P. redivivus nematodes were monoxenised using the described technique above. Four inoculum levels were investigated; 500, 1000, 2000 and 4000 nematodes ml^{-1}. Nematodes were inoculated into flasks containing 10% kidney media as described in the materials and methods section. Flasks were placed in a shaking incubator at 170rpm with a set temperature 22^0C. Sterile samples were taken (using a 1ml Gilson pipette) at intervals for a period of time until the experiment was terminated. Nematodes counts were performed using a 1ml graded counting chamber. Four counts were made for each sample with 4 replicates made for each treatment. Data analysis was undertaken using SpSS one way ANOVA using the test for Least Significant Difference.

Experiment 2

Optimisation of inoculum level of *C. elegans*.

C. elegans nematodes were monoxenised using the above described technique. Four inoculum levels were investigated; 250, 500, 1000, and 2000 nematodes ml^{-1}. Nematodes were inoculated into flasks containing 10% kidney media as described in the materials and methods section. Flasks were placed in a shaking incubator at 170rpm with a set temperature 22^0C. Sterile

samples were taken (using a 1ml Gilson pipette) at intervals for a period of time until the experiment was terminated. Nematodes counts were performed using a 1ml graded counting chamber. Four counts were made for each sample with four replicates made for each treatement. Data analysis was undertaken using SpSS one way ANOVA using the test for Least Significant Difference.

Experiment 3

Temperature optimisation for the growth of *P. redivivus*.

The optimum inoculum level was established in experiment 1 and was used in this experiment to investigate the optimum temperature for growth of the nematode. Six temperatures were investigated; 12^0C, 15^0C, 18^0C, 22^0C, 25^0C and 30^0C. The media used is as described in the materials and methods section. Flasks were placed in a shaking incubator at 170rpm with the set temperatures. Flasks were incubated for an indefinite period of time. The temperature in the incubator was monitored by a data logger. Sterile samples were taken (using a 1ml Gilson pipette) at intervals for a period of time until the experiment was terminated. Nematodes counts were performed using a 1ml graded counting chamber. Four counts were made for each sample with 4 replicates made for each treatement. Data analysis was undertaken using SpSS one way ANOVA using the test for Least Significant Difference.

Experiment 4

Temperature optimisation for the growth of *C. elegans*.

The optimum inoculum level was established in experiment 2 and was used in this experiment to investigate the optimum temperature for growth of the nematode. Four temperatures were investigated; 12^0C, 15^0C, 18^0C and 22^0C. The media used is as described in the materials and methods section. Flasks were placed in a shaking incubator at 170rpm with the set temperatures. Flasks were incubated for an indefinite period of time. The temperature in the incubator was monitored by a data logger. Sterile samples were taken (using a 1ml Gilson pipette) at intervals for a period of time until the experiment was terminated. Nematodes counts were performed

using a 1ml graded counting chamber. Four counts were made for each sample with 4 replicates made for each treatement. Data analysis was undertaken using SpSS one way ANOVA using the test for Least Significant Difference.

Experiment 5

Commercial media evaluation

The growth and development of the nematode *P. redivivus* was evaluated on 5 types of media used on a commercial basis for the production of entomopathogenic nematodes at (AGC, MicroBio Ltd) in fermenters. Flasks of medium were prepared as described in 'The culturing of insect pathenogenic and free living nematodes' in the above. Flasks were placed in a shaking incubator at 170rpm with a the set temperature of 22^0C (described in the results section in Experiment 3). Flasks were incubated for an indefinite period of time. Sterile samples were taken (using a 1ml Gilson pipette) at intervals for a period of time until the experiment was terminated. Nematodes counts were performed using a 1ml graded counting chamber. Four counts were made for each sample with 4 replicates made for each treatment. Data analysis was undertaken using SpSS one way ANOVA using the test for Least Significant Difference. The following media were assessed;

Medium a. *Steinernema* fermenter medium.

% wet weight		
YE	AP	Lecithin
2.66	1.33	1.06

Medium b. *Heterorhabditis* fermenter medium.

% wet weight		
TSB	YE	Lecithin
2.66	1.33	0.33

Medium c. Current *Heterorhabditis* fermenter medium.

% wet weight		
YE	TSB	Bovril
1	2	1.624

Medium d. *Panagrellus redivivus* medium (Cryan, 1963).

% wet weight		
SP	YE	
4	3	

Medium e. Standard flask culture medium.

% wet weight		
Kidney	YE	
10	1	

Medium f 10l aspirator.

% wet weight		
YE	Kidney	Egg
0.78	3.53	5.95

Corn oil was added at the rate of 3.5% to all media, except medium d.

* Medium did not include corn oil.

AP - Animal protein

TSB - Tryptic soya broth

YE - Yeast extract

SP - Soy protein

Experiment 6

Alternative media components

Following the above experiment observing the growth of *P. redivivus* in commercial media. Alternative media were also investigated. Identical culture conditions were used as described in

experiment 5. The following media components were investigated;

Medium containing fish autolysate egg and yeast extract.

% wet weight		
Fish auto-l	Egg	YE
1	3.97	1

Medium containing soya protein isolate, egg and yeast extract.

% wet weight		
Soya	Egg	YE
1	3.97	1

Medium containing egg and yeast extract.

% wet weight		
Egg	YE	
3.97	1	

Medium containing kidney, yeast extract and egg.

% wet weight		
Kidney	YE	Egg
4.7	1	3.97

Medium containing bovril, egg and yeast extract.

% wet weight		
Bovril	Egg	YE
1.62	3.97	1

Medium containing chicken liver, egg and yeast extract;

% wet weight		
Chicken liver	Egg	YE
4	3.97	1

Experiment 7

The use of bovril and optimising the egg component in the medium.

With reference to the results shown in Experiment 6. A medium containing Bovril showed promise for the support of *P. redivivus* in liquid culture. In this experiment the optimum percentage of bovril was investigated for the growth of the nematode. Identical culture conditions were used as described in experiment 5. The following media components were investigated;

Treatment 1.

% dry weight			Wet weight in g per 100ml		
Bovril	Egg	YE	Bovril	Egg	YE
a 0.25	0	2.75	0.41	0	4.2
b 0.25	0.25	2.5	0.41	0.99	3.85
c 0.25	0.75	2	o.41	2.97	3.1
d 0.25*	1	1.75	0.41	3.96	2.7

Treatment 2.

% dry weight			Wet weight in g per 100ml		
Bovril	Egg	YE	Bovril	Egg	YE
a 0.5	0	2.5	0.81	0	3.85
b 0.5	0.25	2.25	0.81	0.99	3.46
c 0.5	0.75	1.75	0.81	2.97	2.7
d 0.5*	1	1.5	0.81	3.96	2.3

Treatment 3.

% dry weight			Wet weight in g per 100ml		
Bovril	Egg	YE	Bovril	Egg	YE
a 0.75	0	2.25	1.22	0	3.5
b 0.75	0.25	2	1.22	0.99	3.1
c 0.75	0.75	1.5	1.22	2.97	2.3
d 0.75*	1	1.25	1.22	3.96	1.9

Treatment 4.

% dry weight			Wet weight in g per 100ml		
Bovril	Egg	YE	Bovril	Egg	YE
a 1	0	2	1.62	0	3.1
b 1	0.25	1.75	1.62	0.99	2.7
c 1*	0.75	1.25	1.62	2.97	1.9
d 1*	1	1	1.62	3.96	1.54

Treatment 5.(Controls)

% dry weight			Wet weight in g per 100ml		
Kidney	Egg	YE	Kidney	Egg	YE
1	0	2	4.7	0	3.1

Experiment 8

Optimisation of the concentration of bovril as a medium component

With reference to the results obtained in Experiment 7, higher concentrations of bovril in the medium were investigated. Identical culture conditions were used as described in experiment 5. The following media components were;

Treatment 1.

% dry weight			Wet weight in g per 100 ml		
Bovril	Egg	YE	Bovril	Egg	YE
a1	1.5	1.5	1.62	5.94	2.3
b1	0.75	2.25	1.62	2.97	3.46
c1	1	2	1.62	3.96	3.1

Treatment 2.

% dry weight			Wet weight in g per 100ml		
Bovril	Egg	YE	Bovril	Egg	YE
a1.5*	1.5	1	2.43	5.94	1.54
b1.5	0.75	1.75	2.43	2.97	1.15
c1.5	1	1.5	2.43	3.96	2.31

Treatment 3.

% dry weight			Wet weight in g per 100ml		
Bovril	Egg	YE	Bovril	Egg	YE
a2*	1.5	0.5	3.24	5.94	0.77
b2*	0.75	1.25	3.24	2.97	1.16
c2*	0.5	1.5	3.24	1.98	2.31

Controls (normal flask diet)

% dry weight		Wet weight in g per 100ml	
Kidney	YE	Kidney	YE
2.12	0.64	10	1

* Denotes remaining cultures.

Experiment 9

Optimisation of the concentration of yeast extract to use in a medium containing bovril.

In the previous experiments it was thought that high levels of yeast extract could be the cause of low population levels. This experiment was designed to investigate various concentrations of yeast extract in the medium. Identical culture conditions were used as described in experiment 5. The following media components were investigated;

Treatments.

% dry weight			Wet weight in g per 100ml		
Bovril	Egg	YE	Bovril	Egg	YE
2	1.5	0	3.24	5.94	0
2	1.5	0.5	3.24	5.94	0.77
2	1.5	1	3.24	5.94	1.54
2	1.5	1.5	3.24	5.94	3.31

Controls (normal flask diet)

% dry weight		Wet weight in g per 100ml	
Kidney	YE	Kidney	YE
2.12	0.64	10	1

Corn oil was added at the rate of 3.5% or 3.5g per 100ml.

Experiment 10

10L fermenter culture of *P. redivivus*.

With reference to the results obtained in experiment 9, a medium was prepared for use in a 10 L fermenter. These results indicated that a medium containing 2% bovril, 1.5% egg and 1% yeast extract supported good growth and development and was therefore selected for use in a 10 L fermenter for growing *P. redivivus*. The medium was prepared as described above and placed in a 10 L glass vessel in which it was autoclaved. Cooled medium was then pumped into a sterile 10 L air lift fermenter and inoculated with *E. coli*. The medium was then incubated at 21^{0}C (the 10 litre vessel was held in a constant temperature room and therefore the temperature was set at 21^{0}C and could not be changed) for 24 h. Sterile samples were taken (using a 1ml Gilson pipette) at intervals for a period of time until the experiment was terminated. Nematodes counts were performed using a 1ml graded counting chamber, 4 counts were made for each sample. Data analysis was undertaken using SpSS one way ANOVA using the test for Least Significant Difference with 95% confidence limits.

RESULTS

Experiment 1

Optimum inoculum level for *Panagrellus redivivus*

Experiment 1 investigated the population growth of *P. redivivus* at 4 different inoculum levels. Nematodes were cultured at 22^{0}C for an unlimited period of time in a base medium described in the 'materials and methods'. Four replicates for each treatment were used. Inoculum levels of 500, 1000, 2000, and 4000 nematodes ml^{-1} were investigated. Table 3 shows the population growth of *P. redivivus*, the results indicate that higher inoculum levels give higher nematode counts from days 2 to 7. Maximum yields of nematodes were obtained at day 11 (Fig. 1), the highest obtained from an inoculum level of 4000 nematodes ml^{-1} (mean = 280 000 ml^{-1}). Analysis of variance showed that inoculum level of 4000 nematodes ml^{-1} was significantly different at p=0.05 level from the other treatments at day 11 when maximum population levels were obtained for all treatments. There was no significant difference for inoculum levels 500, 1000 and 2000 on day 11. It was also observed that the population growth of nematodes from treatment inoculum level of 4000 nematodes ml^{-1} was significantly superior throughout the experiment except on day 6.

The hypothesis for this experiment was that the different inoculum levels might have required different times to reach maximum population levels. A lower inoculum level would be harvested later but the maximum population level would be the same as higher inoculum levels. However all inoculum levels reached maximum yields on the same day even though there were wide differences in the nematode numbers at the beginning of the experiment. It was also observed that after day 11 populations of nematodes in all treatments declined.

Table 3 . Mean nematode counts for *P. redivivus* (four replicates for each treatment). Four inoculum levels tested.

Day	500	1000	2000	4000	Significance
2	1250.00	2625.00	3812.50	12062.50*	*
4	11875.00*	65250.00	43500.00	81750.00*	**
6	55375.00*	153750.00	153000.00	168750.00	*
7	66125.00	115750.00	157250.00	185500.00	Ns
11	243500.00	255750.00	229250.00	280000.00*	*
16	123000.00*	164250.00	160250.00	234500.00*	**
18	84750.00*	105000.00	127500.00	157750.00*	**

Experiment 2

Optimum inoculum level for *Caenorhabditis elegans*.

Experiment 2 shows the optimum inoculum level for *C. elegans*. The results (see Table 4) demonstrate that that the inoculum levels 250 and 500 gave the highest yields (Fig. 2) compared to inoculum levels 1000 and 2000 nematodes per ml. However the inoculum levels 250, 500 and 1000 are significantly different from 2000 at the 5% level but not from each other.

Table 4. Mean counts of nematode for *C. elegans* (four replicates for each treatment). Four incoculum levels tested.

Day	250	500	1000	2000	Significant
3	23000.00	49000.00	81000.00	159000.00	
6	328000.00*	400000.00*	261000.00	290000.00	**
8	560000.00*	544000.00*	350000.00	311000.00	**
10	630000.00*	640000.00*	314000.00	335000.00	**
13	528000.00*	595000.00*	396000.00	413000.00	**
15	528000.00*	552000.00*	443000.00	381000.00	**

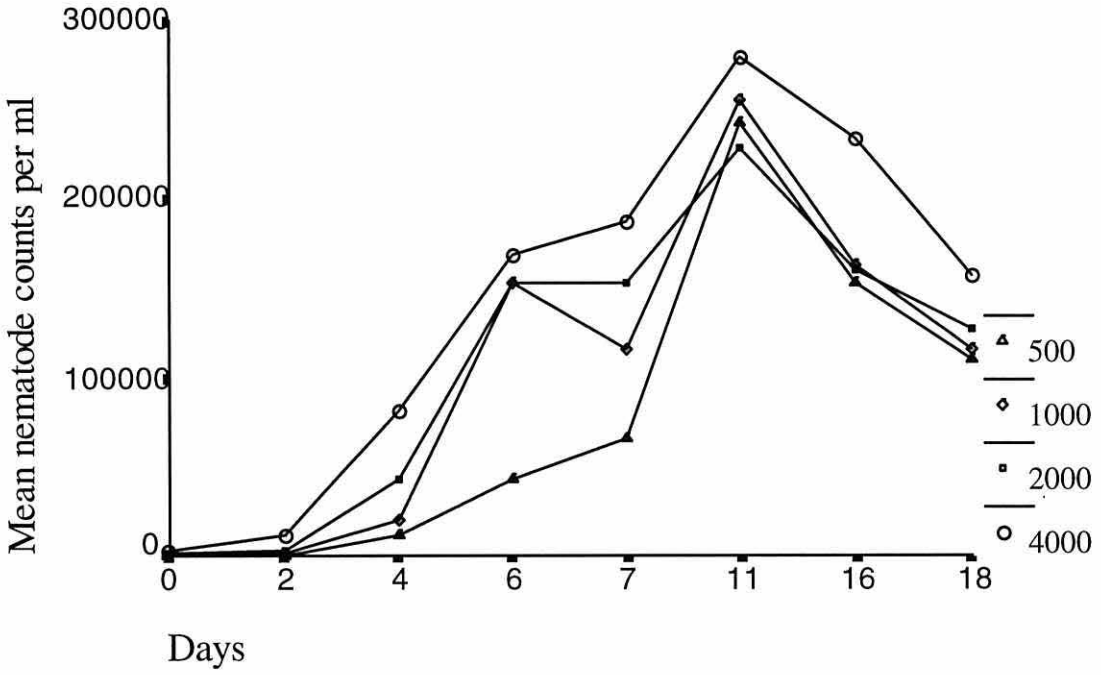


Figure 1. Four inoculum levels were investigated to observe optimum growth of the nematode *P. redivivus* in liquid culture. The inoculum levels investigated were 500, 1000, 2000 and 4000 nematodes ml^{-1}.

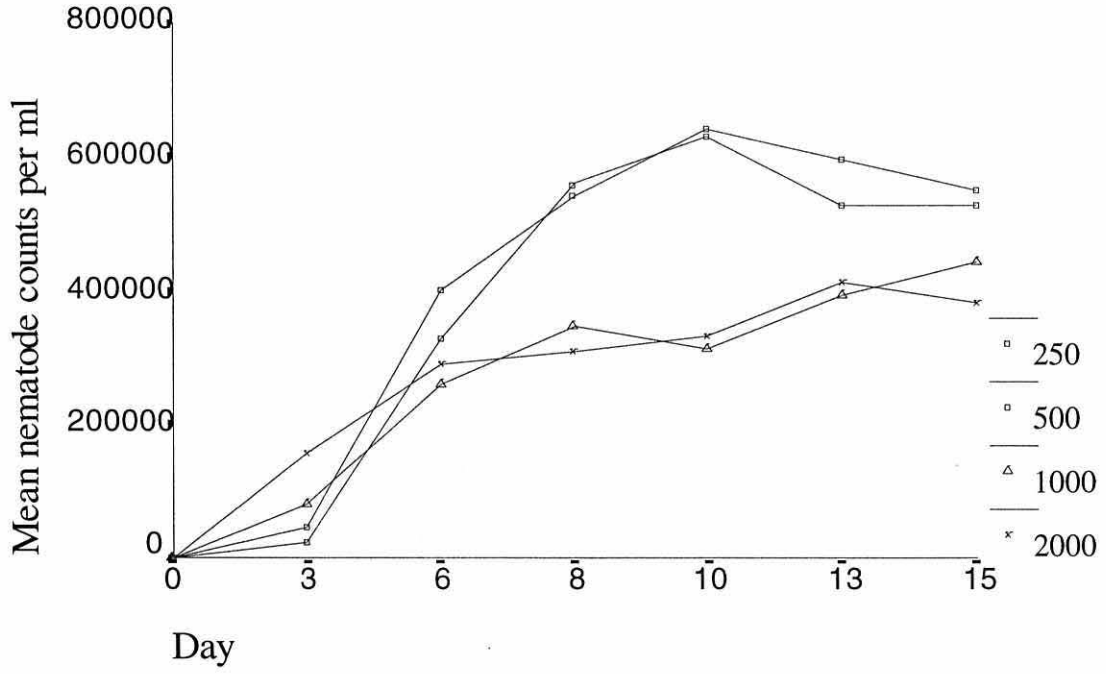


Figure 2. Four inoculum levels were investigated to observe optimum growth of the nematode *C.elegans* in liquid culture. The inoculum levels investigated were 250, 500, 1000 and 2000 nematodes ml^{-1}.

Experiment 3.

Temperature optimisation for *P. redivivus*.

The objective of experiment 3 was to observe the optimum growth of *P. redivivus* at different temperatures in liquid culture. The optimum inoculum level for this species was established previously and was used in this experiment. For *P. redivivus* 6 temperatures were investigated; 30°C, 25°C, 22°C, 19°C, 15°C and 12°C, 4 replicates were used for each treatment.

The results shown in Table 5 demonstrate that the fastest growth and highest population numbers were obtained for the temperatures 25°C and 30°C. Peak populations occurred on day 14 and 9 respectively. These temperatures were significantly different from each other and the other treatments at the 5% level and remained so throughout the experiment. However nematodes grown at these high temperatures were pale and thin and did not have the typical sinusoidal movement. From the results 18°C and 22°C are the optimum temperatures for growth producing approximately 118 000 and 127 000 nematodes per ml respectively. Nematode populations were significantly different at 22°C at the 5% level from the nematodes grown at 12°C,15°C and 19°C which showed no significant difference between each other. Nematodes grown at 12°C took the longest time to reach peak populations, 31 days (Fig.3).

Table 5. *P. redivivus* incubated at six different temperatures to investigate optimum growth (mean of 4 replicates).

Day	12°C	15°C	18°C	22°C	25°C	30°C	Significant
0	2000.00	2000.00	2000.00	2000.00	2000.00	2000.00	
3	1650.00	4150.00	23625.00	25925.00	42487.50	41995.00	**
9	5875.00	35375.00	48125.00	78250.00	99500.00	165500.00	**
14	27000.00	70500.00	85750.00	85500.00	158750.00	133000.00	**
20	216000.00	92750.00	109000.00	126750.00	107500.00	28750.00	**
28	73000.00	97250.00	118750.00	103000.00	19750.00	0.00	**
31	11750.00	105500.00	116750.00	112750.00	0.00	0.00	**
42	88500.00	100750.00	80500.00	27200.00	0.00	0.00	**

It can be concluded that the results indicate that the higher temperatures produce the faster nematode growth, but are followed by rapid population collapse.

Experiment 4

Temperature optimisation for *C.elegans*.

The objective of experiments 4 was to observe the optimum growth of *C.elegans* at different temperatures in liquid culture. The optimum inoculum level for this species was established previously and was used in this experiment. For *C.elegans* 4 temperatures were investigated; 22^{0}C, 19^{0}C, 15^{0}C and 12^{0}C 4 replicates were used for each treatment.

Table 6. *C. elegans* incubated at six different temperatures to investigate optimum growth (mean of 4 replicates).

Day	12^{0}C	15^{0}C	19^{0}C	22^{0}C	Significant
0	500.00	500.00	500.00	500	
3	2687.50	163375.00	179750.00	182000.00	**
9	73500.00	203000.00	387500.00	373000.00	**
14	40187.50	207000.00	377000.00	385500.00	**
17	232000.00	74562.50	465000.00	472000.00	**
28	218000.00	87750.00	374000.00	386500.00	**
32	210000.00	333250.00	465500.00	345750.00	**

It can be seen from the results (seeTable 6) that *C. elegans* grew faster and reached peak populations faster at the temperatures 19^{0}C and 22^{0}C. Throughout the experiment nematodes grown in flasks kept at 19^{0}C and 22^{0}C showed no significant difference at the 5% level between each other. There was a significant difference at the 5% level between flasks held at 22^{0}C and 19^{0}C compared to 12^{0}C and 15^{0}C. There were still many mixed developmental stages in the cultures of nematodes grown at 15^{0}C and 12^{0}C when the experiment was terminated. However approximately 85% of nematodes counted were dauer nematodes in the cultures grown at 19^{0}C and 22^{0}C. At day 28 nematodes grown at 22^{0}C showed a decline in numbers whereas nematodes grown at 19^{0}C persisted at populations of 500 000 nematodes per ml beyond 28 days (Fig.4).

Higher temperatures produced faster growth rates and higher population levels. However the optimum temperature in this experiment was 19^{0}C as this allowed nematodes to persist longer in the medium. For *C. elegans* peak population levels were obtained at 19^{0}C with the formation of dauer stages (resting stage larva induced by unfavourable conditions). This temperature appears to be the optimum temperature for growth and development.

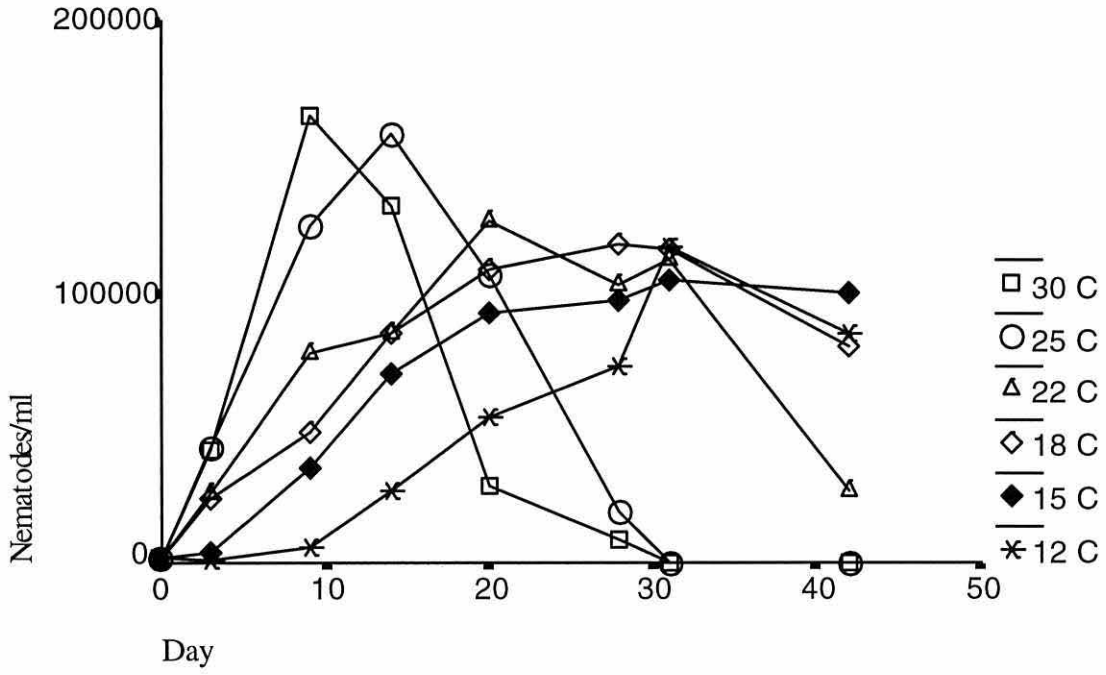


Figure 3. The growth of *P. redivivus* at six different temperatures. After 28 days nematodes grown at 25^{0}C and 30^{0}C, rapidy declined in numbers.

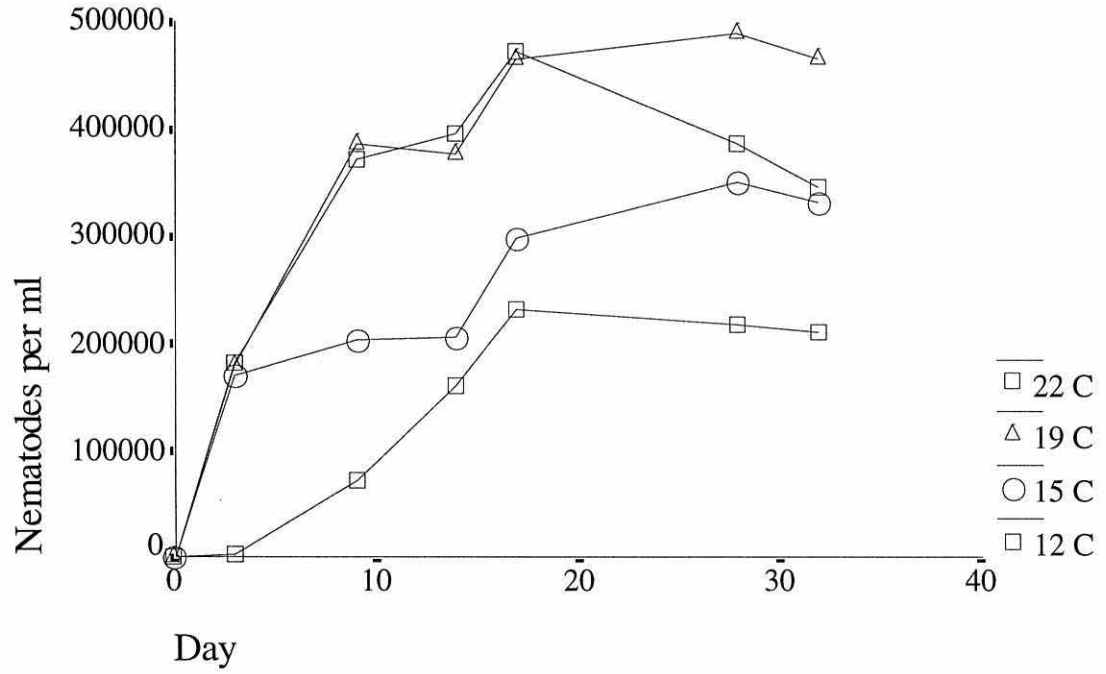


Figure 4. The growth of C. elegans at 4 different temperatures.

Experiment 5

Media optimisation for *P. redivivus*.

The results shown Fig. 5 demonstrates that the medium producing highest yields of nematodes was medium (e) containing 10% Kidney , 3.5% corn oil, 1% yeast extract. The maximum yield was 230 000 nematodes ml^{-1}. There was a highly significant difference for maximum yield at the P=0.05 level compared to all the other media. A medium containing 4% soya peptone failed to sustain nematode populations beyond day 7.

Experiment 6

Fig. 6 demonstrates *P. redivivus* grows very well on all 6 types of media investigated. The optimum growth was obtained from nematodes grown on the medium containing chicken liver. This medium averaged 180 000 nematodes per ml at the highest population level at day 22. Throughout the experiment nematode population levels remained significantly higher in the chicken liver medium than in other media at the 5% level (see Table7). Nematodes from this medium appeared extremely active, healthy and dark.

The medium containing bovril also produced interesting results. Towards the end of the experiment population levels reached approximately 170 000 nematodes ml^{-1}. There was no significant difference between population values on day 22 for nematodes grown on bovril or chicken liver. Nematodes grown in bovril also appeared very healthy and dark.

All the media types supported good growth of the nematode *P. redivivus*. However chicken liver and bovril supported the better growth producing a higher number of individuals and these media were investigated further. The media containing fish autolysate, soya and egg could be considered as good growth media as population numbers reached approximately 100 000 nematodes per ml. However when the experiment was carried beyond 22 days at which point populations began to rapidly decline for nematodes grown on fish autolysate, soya isolate, egg and kidney. Nematodes grown on bovril and chicken liver were more persistent in the medium.

Table7 . *P. redivivus* grown on 6 different media. Mean counts for 4 replicates.

Day	Fish auto	Soya	Egg	Kidney	Bovril	Chicken	Significant
0	2000	2000	2000	2000	2000	2000	
4	3525	2925	4000	4300	3350	6825	
8	44938	53313	48250	51813	52813	59875	
12	80500	94375	83750	95750	73875	126750	
16	87000	107250	99750	126750	109250	153750	
20	115500	91000	108500	116000	108000	154000	
22	80000	97300	115000	138000	171500*	183000*	**

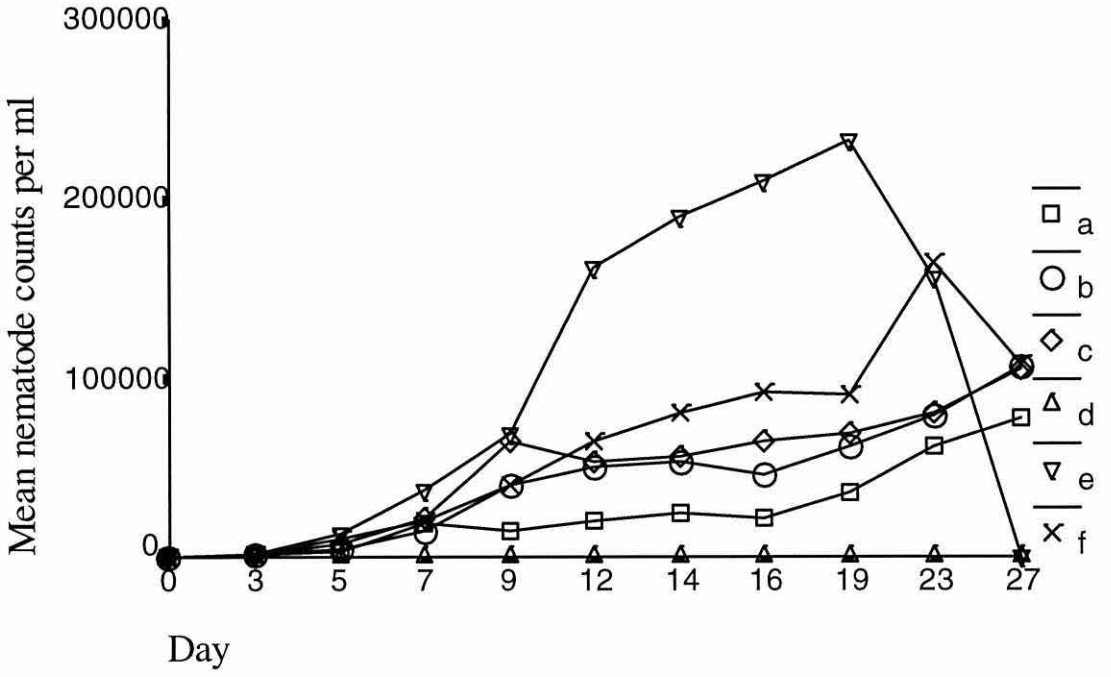


Figure 5. Growth of *P. redivivus* in 5 commercial media: a = *Steinernema* medium, b = *Heterorhabditis* medium, c = Current *Heterorhabditis* medium, = *Panagrellus redivivus* (Cryan, 1963), e = Standard flask culture medium, f = 10 l aspirator medium.

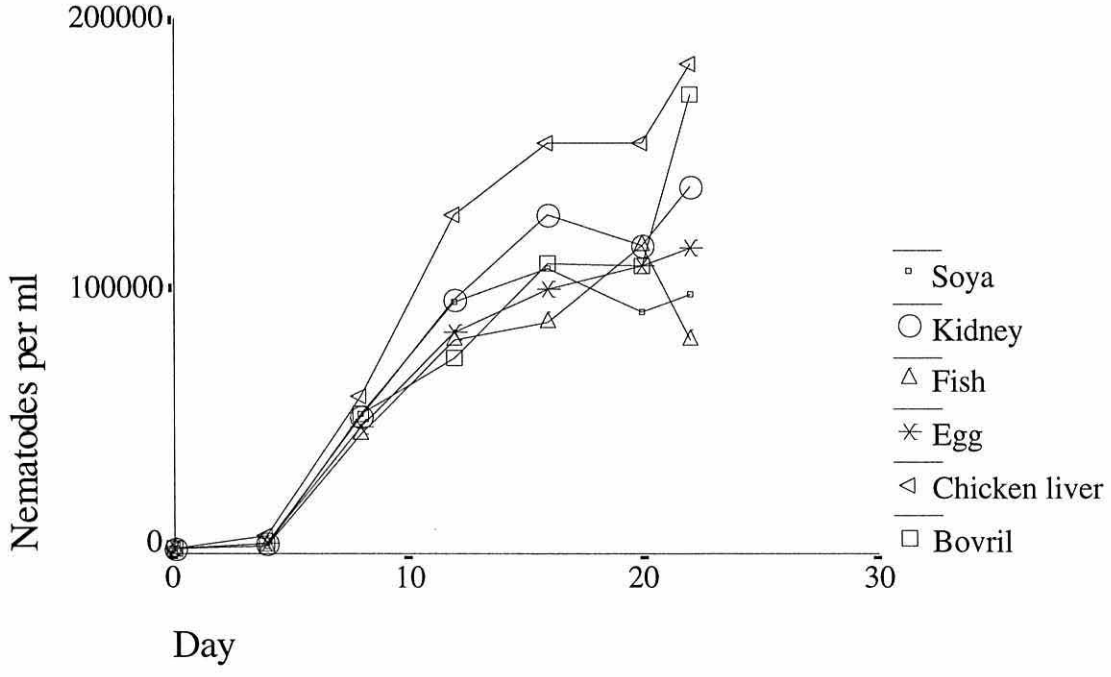


Figure 6. Growth of *P. redivivus* in media containing; fish autolysate, soya protein, egg and yeast extract, kidney, bovril and chicken liver.

Experiment 7

With reference to the results shown in Experiment 6. A medium containing Bovril showed promise for the support of *P. redivivus* in liquid culture. In this experiment the optimum percentage of bovril was investigated for the growth of the nematode. In Fig.7 after 7 days of culture the following treatments remained; Controls, 1d,2d,3d, 4c and 4d. The other treatments were unable to support growth of *P. redivivus*. Until day 10 of culture the populations in the controls were significantly higher than the remaining treatments at the 5% level. Treatment 1d was not significantly different from the control on day 10. All treatments were not significantly different from each other or the control on day 15. Towards the end of the experiment treatment 3d contained populations that were significantly higher than the remaining treatments. The maximum population numbers recorded were from treatment 1d (86000 per ml) on day 10 (see Table 8).

Results demonstrate it is possible using a medium containing 0.25% bovril, 1% egg and 1.75% YE (dry weight) to produce population numbers equivalent to a medium containing kidney. However in all treatments high numbers of juveniles were observed and did not develop into adults towards the end of the experiment. This could be due to the fact that the nutrient levels were depleted in the medium. Normally when using a medium containing bovril 4% solids are used (Godliman et al, pers. comm). In all the successful treatments the highest egg percentage dry weight was used, and hence the increased percentage of egg may have the effect of promoting growth. However almost all the successful treatments had the lowest dry weight of yeast extract used. Increasing the yeast extract concentration may have the effect of inhibiting the growth of the nematode. The best treatment containing 0.25% bovril, 1% egg and 1.75% YE contains a good ratio of egg to YE.

Table 8 . *P. redivivus* grown in media with varying bovril concentration.

Day	Control	4c	1d	3d	2d	4d
0	2000.00	2000.00	2000.0	2000.00	2000.00	2000.00
3	6266.67	1533.00	5000.00	2400.00	1800.00	6733.33
7	58833.33	1466.67	34833.33	3200.00	0.00	35333.30
10	78500.00	3960.00	87800.00	5533.00	0.00	9936.00
15	36400.00	0.00	204000.00	19866.67	0.00	23200.00
22	11200.00	0.00	12333.33	23466.67	0.00	19666.67

Experiment 8

With reference to the results obtained in Experiment 7, higher concentrations of bovril in the medium were investigated. Identical culture conditions were used as described in experiment 5. The following media components were;

Treatment 1.

% dry weight			Wet weight in g per 100 ml		
Bovril	Egg	YE	Bovril	Egg	YE
a1	1.5	1.5	1.62	5.94	2.3
b1	0.75	2.25	1.62	2.97	3.46
c1	1	2	1.62	3.96	3.1

Treatment 2.

% dry weight			Wet weight in g per 100ml		
Bovril	Egg	YE	Bovril	Egg	YE
a1.5*	1.5	1	2.43	5.94	1.54
b1.5	0.75	1.75	2.43	2.97	1.15
c1.5	1	1.5	2.43	3.96	2.31

Treatment 3.

% dry weight			Wet weight in g per 100ml		
Bovril	Egg	YE	Bovril	Egg	YE
a2*	1.5	0.5	3.24	5.94	0.77
b2*	0.75	1.25	3.24	2.97	1.16
c2*	0.5	1.5	3.24	1.98	2.31

Controls (normal flask diet)

% dry weight		Wet weight in g per 100ml	
Kidney	YE	Kidney	YE
2.12	0.64	10	1

*** Denotes remaining cultures.**

After 3 days of culture the following treatments remained in (Fig.8); Controls, 2a,3a,3b and 3c. The other treatments were unable to sustain growth of the nematodes. This may have been due to the high concentration of yeast extract.

Throughout the experiment the control mean population numbers were significantly higher at the 5% level compared to all the other treatments. Populations reached a maximum mean of 170000 per ml for the controls. Treatment 3a showed significantly higher population levels than treatments 2a, 3b and 3c. The maximum mean population yield obtained for treatment 3a was 108 000 nematodes per ml.

From the results it is clear that a superior growth is obtained with the controls containing the conventional flask diet. However a medium 3a containing 2% bovril, 1.5% egg and 0.5% yeast extract (dry weight) supported growth and development of the nematode and produced sufficient numbers in the culture (108000 per ml). This is also an improvement on the results obtained from the previous experiment using a medium containing 0.25% dry weight bovril. In both the treatments 2a and 3a nematodes were observed to be very active and dark.

It was also observed that the most successful treatments also had the lowest yeast extract concentration and highest bovril concentration. It would therefore be necessary to investigate whether higher concentrations of bovril stimulated any increase in population levels. Samples of nematodes were taken from each treatment and freeze dried for lipid analysis to compare the nutritional value of nematodes grown on a kidney medium to that of nematodes grown on a diet containing bovril.

Table 9. Growth data of P. redivivus in media containing higher concentrations of bovril.

Day	Control	2a	3a	3b	3c	Significant
0	2000	2000	2000	2000	2000	
3	37800	21550	33500	1850	4050	
6	77250	50500	65125	1300	5250	
10	120000	43312.50	79700	900	4100	
13	129500	45000	79700	1000	3300	
17	144250	67100	108500	3150	17250	
23	170750	74125	71250	10300	16275	
31	170500	42375	66750	36750	32300	

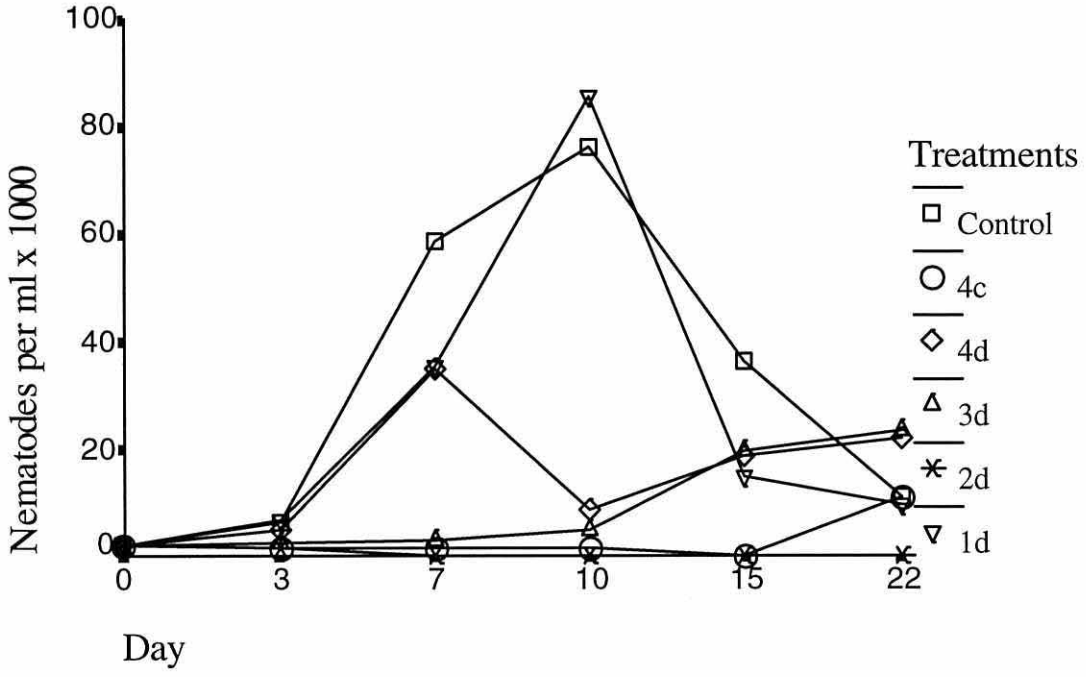


Figure 7. Growth of *P. redivivus* in media containing dry weight components: 4c = 1% bovril, 0.75% egg, 1.25%; 4d = 1% bovril, 1% egg, 1% YE; 3d = 0.75% bovril, 1% egg, 1.25% YE; 2d = 0.5% bovril, 1% egg, 1.5% YE; 1d = 0.25% bovril, 1% egg, 1.75% YE; Control = 2.12% kidney, 0.64% YE.

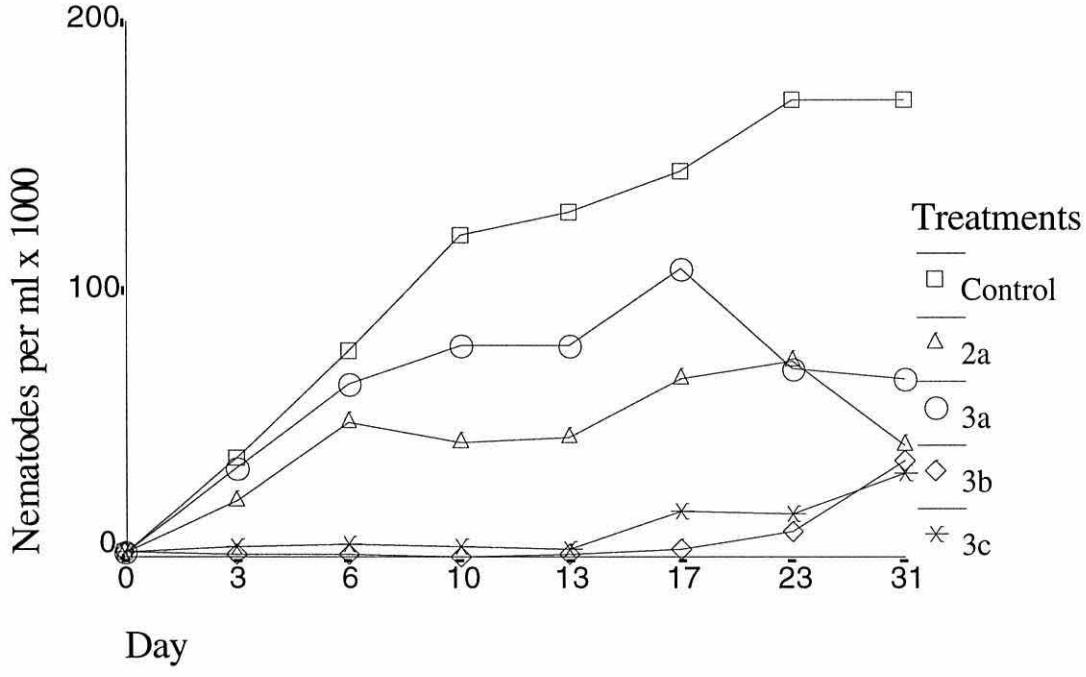


Figure 8. Growth of *P. redivivus* in media containing higher concentrations of bovril: 2a = 1.5% bovril, 1.5% egg, 1% YE; 3a = 2% Bovril, 1.5% egg, 0.5% YE; 3b = 2% bovril, 0.75% egg, 1.25% YE; 3c = 2% bovril, 0.5% egg, 1.5% YE; Control = 2.12% kidney, 0.64% YE (dry weight).

Experiment 9

In the previous experiments it was thought that high levels of yeast extract could be the cause of low population levels. This experiment was designed to investigate various concentrations of yeast extract in the medium. Identical culture conditions were used as described in experiment 5. The following media components were investigated;

Treatments

% dry weight			Wet weight in g per 100ml		
Bovril	Egg	YE	Bovril	Egg	YE
a2*	1.5	0.5	3.24	5.94	0.77
b2*	0.75	1.25	3.24	2.97	1.16
c2*	0.5	1.5	3.24	1.98	2.31

Control

% dry weight		Wet weight in g per 100ml	
Kidney	YE	Kidney	YE
2.12	0.64	10	1

Fig.9 shows that the control treatment produced the highest population numbers and is significantly different at the 5% level throughout the experiment compared to the other treatments. The treatment containing 1.5 % yeast extract produced the lowest population levels and unhealthy nematodes and was significantly different from all the other treatments throughout the experiment. On day 15 the treatment containing 1 % yeast extract had a higher population level and was significantly different at the 5% level from treatments containing 0%, 0.5% and 1.5% yeast extract.

From these results it appears that a medium containing 1.5% yeast extract or more is detrimental to the growth of *P. redivivus*. The optimum concentration of yeast extract to be included in a medium containing 2% bovril and 1.5% egg is 1% yeast extract. The maximum population average obtained with this treatment was 132 000 nematodes per ml. Nematodes were observed to be very healthy and dark indicating adequate fat reserves. Therefore a medium containing

2% bovril, 1.5% egg and 1% yeast extract was selected for use in a 10L fermenter for growing *P. redivivus.*

Table 10. *P. redivivus* grown in medium containing varying levels of yeast extract.

Day	Control	0 Yeast	0.5 Yeast	1 Yeast	1.5 yeast	Significant
0	2000	2000	2000	2000	2000	*
5	86100	23100	79500	76500	3050	*
8	77000	51100	65500	71000	4050	*
12	166000	82750	79250	82750	5250	*
15	196750	88250	77000	132000	5100	*
19	180500	74000	68250	95750	1490	*
21	163250	60750	54625	78250	2247	*

Experiment 10

With reference to the results obtained in experiment 9, a medium was prepared for use in a 10 liter fermenter. These results indicated that a medium containing 2% bovril, 1.5% egg and 1% yeast extract supported good growth and development and was therefore selected for use in a 10L fermenter for growing *P. redivivus.* Fig. 10 shows that the medium supported growth and development of *P. redivivus* in a 10L fermenter. High yields were obtained and the nematodes produced were very active dark and healthy. Table 11 shows the nematode population numbers obtained from the fermenter medium.

Table 11. *P. redivivus* grown in a 10L fermenter in medium containing 2% Bovril, 1.5% egg and 1% yeast.

Day	0	4	11	14	18	25	27
Mean of 4 counts	2000.00	2456.00	3024.00	5622.00	8976.00	18223.00	13843.00

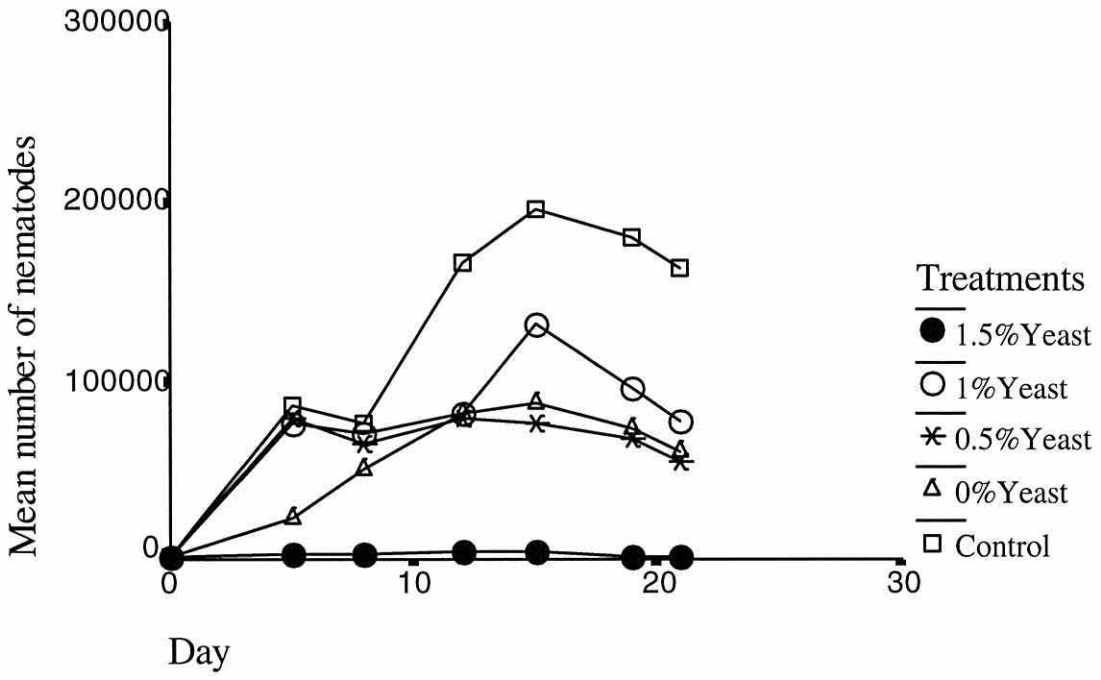


Figure 9. Optimisation of the concentration of YE using 2% bovril in media containing dry weight components: YE= 0%, 0.5%, 1% and 1.5%; Control = 2.12% kidney, 0.64% YE (dry weight).

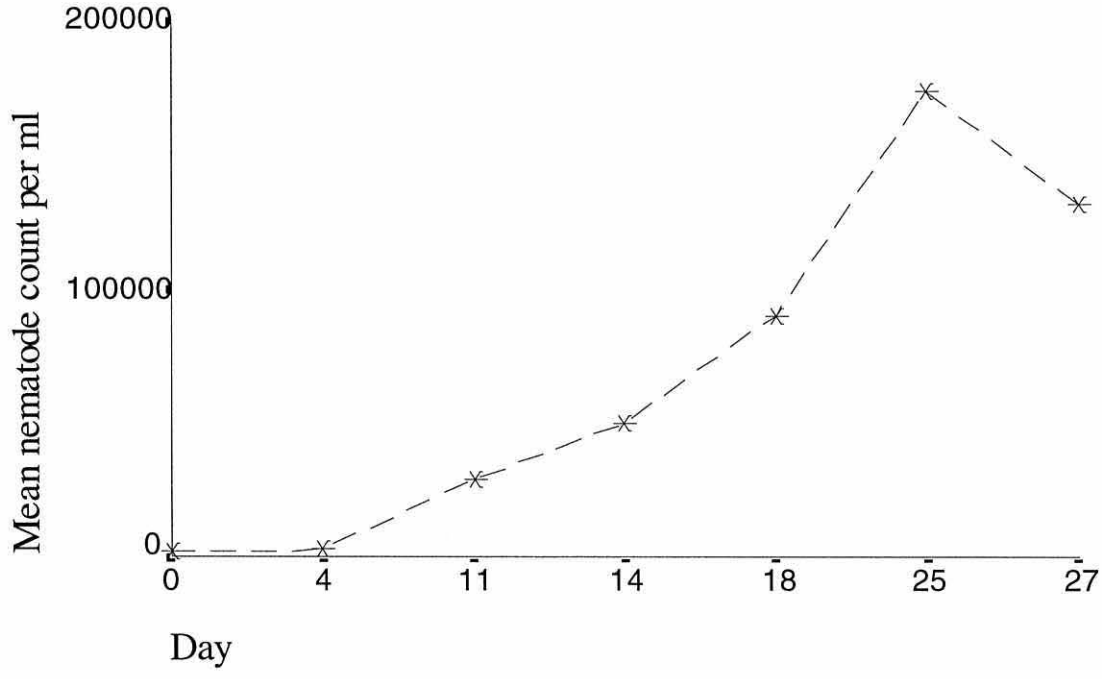


Figure 10. Growth of *P. redivivus* in a 10 L fermenter in medium containing 2% bovril, 1.5% egg and 1% YE.

DISCUSSION.

Optimum inoculum level

The objective of these experiments was to find the optimum initial inoculum level for *Panagrellus redivivus* and *Caenorhabditis elegans* providing the maximum population levels whilst producing good quality nematodes. In experiment 1 inoculum levels of 500, 1000, 2000 and 4000 nematodes ml^{-1} range were investigated. The hypothesis for this experiment was that different inoculum levels require different times to reach maximum population levels and an overall effect on the yield would be obtained. A low inoculum level would be harvested later but the overall yield would be the same as the high inoculum levels. However from the results all inoculum levels reached peak populations on or around the same day, even though there was a significant difference in overall numbers obtained. This indicates that initial inoculum level has a profound effect on yields. The conclusion from experiment 1 is that an inoculum level of 4000 nematodes per ml is required to produce maximum yields after 11 days. The reason for this may be due to the fact that the nematode *P. redivivus* is not hermaphroditic and require the presence of males and females in the culture medium. Lower inoculum levels may result in lower reproduction rates i.e. less males and females coming into contact with each other. Exploration of inoculum levels above 4000 ml^{-1} was not feasible as the objective was to find the minimum effective inoculum level that is also economically viable on a larger scale for the inoculation of 1000L fermenters. Therefore the results demonstrate that inoculum levels for *P. redivivus* between 500 and 2000 could be used effectively.

It can be concluded that high inoculum levels produce a rapid population rise and inoculum levels for example of 500 nematodes ml^{-1} will take longer to reach maximum population levels (approximately 20 days compared to 11 to 14 days for an inoculum level of 4000 nematodes ml^{-1}). However nematodes grown from the lower inoculum levels tend to persist longer in the culture medium after reaching the maximum yields. They also appeared darker indicating (J Wijbenga pers. comm.) a higher lipid content and generally were of a better quality. Cultures from lower inoculum levels had more adults present at the the maximum yields and remained dark and healthy even after reaching maximum population yields. There has been very little published information on the required inoculum level for *P. redivivus* in culture. Wilkenfeld et

al (1984) mentions an initial stocking density of 2,500 nematodes cm^{-2} on a solid medium substate. As research work has not been undertaken on the liquid culture of *P. redivivus* until now, this is the first indication of the importance of the initial inoculum level.

A similar experiment was undertaken with *C. elegans* using the test inoculum levels of 250, 500, 1000 and 2000 nematodes ml^{-1}. It is concluded from the results that inoculum levels of 250 and 500 nematodes ml^{-1} gave maximum yields producing nematodes that are healthy and dark. This nematode is a hermaphrodite only producing males under certain environmental conditions. A dauer stage is formed under certain environmental conditions which are not conducive to growth i.e. depletion of food reserves, high concentration of excretory products and low O_2 tensions. It is possible that the higher inocululm levels may have induced the unfavourable conditions in the flasks preventing maximum yields and not all of the initial inoculum nematodes left the dauer stage at the higher inoculum levels. Gbewonyo et al, (1994) demonstrated that the pH of the liquid medium shows a gradual drift upwards, presumably as a result of excretion of waste products into the medium, producing unfavourable conditions for growth at higher population numbers. Work by Golden and Donald, (1984) suggests that environmental cues such as food availability and temperature influence the production of a dauer inducing pheromone. The pheromone is a measure of population density and induces the formation of the dauer stage when conditions become unfavourable.

Optimum temperature

The results are as expected in that *P. redivivus* grows faster and produces higher population numbers at higher temperatures. From the results it is clear that highest population levels are obtained with 30^0C and 25^0C. Peak populations were reached at day 10 and 14 respectively. Although higher temperatures produced a faster reproduction rate the quality of the nematodes produced was much reduced. Nematodes appeared pale and thin at peak population levels at 30^0C and 25^0C, and few adults were seen. A rapid decline in population numbers was also observed. The optimum temperature for growth concluded from this experiment is either 18^0C or 22^0C. Both temperatures produced peak populations between days 20 and 30. The temperature 22^0C has been chosen for future experiments due to the faster reproduction rate and of nematodes which were dark and healthy producing many adults.

For *C. elegans* peak population levels were obtained at 19^{0}C with the formation of dauer stages. The formation of the dauer stages and the lower population levels recorded at 15^{0}C and 12^{0}C are in agreement with the findings by Golden and Riddle (1983). Their work demonstrated that incubation temperatures above 20^{0}C result in an increased percentage of a population forming dauer larvae is produced when other environmental parameters were kept constant but temperature reductions from 25 to 15^{0}C will induce the dauer larvae to recover.

Media development

Results obtained in chapter 5, and the work by (Plagman, 1939; Nikolsky,1963; Thomson, 1963, 1966; Gerlach and Schrage, 1969; Vallet et al, 1970; Opuszynsk, 1977; Rychly, 1977; Kahan et al,1975, 1979, 1980, 1983, 1984; Hofsten et al, 1983; Wilkenfeld et al, 1984; Biedenbach, 1989; Rottman et al, 1991), demonstrates that *P. redivivus* is the most successful feed replacement in shrimp trials and therefore further optimisation work was concentrated on this nematode. Having established optimum initial inoculum level and temperature an optimal medium was developed. Initially 6 media types used on a commercial basis at AGC, MicroBio Ltd were investigated to culture *Steinernema feltiae* and *Heterorhabditis megidis* on a large scale. It was clear from the results obtained that *P. redivivus* grew and developed most successfully on a medium with a nitrogen source containing kidney. However this medium is used as a 'never fail' medium for culturing unknown nematodes, use on a large scale is not feasible as it is very expensive. Fig.7 showed that nematodes developed successfully on medium b, c and f. However b and c contain mostly dry ingredients which are easier and cheaper to use than the medium f which contained fresh egg. It is concluded from this experiment that medium c containing Bovril, TSB and YE is adequate to produce nematodes. The nitrogen source came from bovril and is cheaper than either egg or kidney.

Other nitrogen sources were also investigated to see if they could improve on the overall yield. Chicken liver was superior to the other treatments and the control in supporting growth and development. However use of this medium on a large scale is not feasible as it requires the presence of an enzyme to break up the connective tissue of the chicken liver and to handle this ingredient in large quantities is awkward. Medium containing bovril again supported good

growth and produced nematodes that were active and dark. Hence possibility of manipulating the bovril component of the medium to improve the overall yields was also explored. The results indicate that some combinations of yeast extract and bovril may be detrimental to the growth of the nematodes. However a medium containing 0.25% bovril, 1% egg and 1.75% yeast extract produced populations equal to those obtained from the control on an equal dry weight ratio basis. However in all treatments approximately 90% of the population were juveniles towards the end of the experiment. This may have been due to the depletion of the nutient supply. In all successful treatments the highest percentage of egg was used. The percentage of egg used may have the effect of promoting growth and it's presence may have the effect of counteracting the detrimental effect of the the yeast extract in high concentrations. Almost all successful treatments apart from 1d had high concentrations of egg and the lowest dry weight of yeast extract used. As increasing the concentration of yeast extract may have an inhibiting effect on the growth of nematodes. This was investigated, superior growth was obtained for the control treatment containing kidney (Fig.10), a medium containing 2% bovril, 1.5% egg and 0.5%yeast extract supported good growth and development and produced enough nematodes for the medium to be considered for use in a larger scale culture vessel (108 000 nematodes per ml^{-1}). The nematodes appeared dark and healthy. It was observed that the most successful treatments that supported good growth and development had the lowest yeast extract concentration and the highest bovril concentration.

The optimum concentration of yeast extract was then established (Fig.11) and maximum population yields were obtained in a medium containing 1% yeast extract. If the concentration of yeast extract is increased this appears to have a detrimental effect on the nematodes. The optimum inoculum level, temperature and medium composition was established and therefore may be applied to growth of this nematode on a large scale. As (Fig.12) shows that *P. redivivus* can be grown successfully in a 10L fermenter using the new medium. After 25 days of incubation approximately 180 000 nematodes ml^{-1}were obtained in the fermenter culture.

The general method for culturing *P. redivivus* for fish and shrimp larval feeds has been on a moist paste in shallow containers in a non sterile environment (Watanabe et al., 1980; Fontaine et al., 1982; Kahan, 1984/1985; Biedenbach, 1989; Radwin and Rouse, 1990; Rouse et al, 1992).

Various substrates have been investigated by the authors including; oatmeal, oatmeal and agar, masa harina flour, pinto beans, whole wheat flour, high protein cereal, torulose yeast, lentils, green peas, dried cheese whey and cooked rolled oats. Currently mass culturing the quantities of nematodes needed at the later substages for penaeid larviculture to obtain growth equal to that of Artemia is the major limiting factor for commercial applications. Biedenbach et al., (1989) reported producing 6000cm^{-2} on a solid medium and Fontaine et al., (1982) reported culturing up to 11000 nematodes cm^{-2} on an oatmeal medium. The quantities required for commercial applications far exceed those that can be produced on a solid medium.

The advantages of culturing *P. redivivus* in liquid culture under defined conditions as a result of present work far out weighs previous methods used. Large volumes can be produced in fermenting vessels, i.e. if 1,800 000 nematodes ml^{-1} can be produced in a 10L fermenter then if production is increased to a 1000L fermenter, nematodes can be produced in numbers in the order of 108 x 10^9 nematodes per fermenter. Material costs for feeding a 20 000 litre larval rearing tank with 3 *Artemia* nauplii ml^{-1} per day is about $30. The estimated cost of feeding *P. redivivus* at 70ml^{-1} per day is $20 based on the supermarket price of Masa Harina and a 10 day nematode culture lifespan Wilkenfeld, (1984). Compared to the estimate for the feeding rate of 70 nematodes ml^{-1} (Wilkenfeld 1984), the cost of raw materials required for the production of *P. redivivus* in a 1000L fermenter is approximately $225 and therefore a 10 day fermentation culture would cost approximately $30 per day. However a 1000 L fermenter is capable of producing 108 x 10^9 nematodes which if the application rate is between 60 -70 ml^{-1}, would provide between 70-80 applications for a 20, 000 litre larval rearing tank.

With the development of a liquid medium of defined components that are cheap and easy to use production will become economically viable on a larger scale. The use of a defined medium allows the production of nutritionally uniform nematodes. The removal of nematodes from the culture medium is made simpler by the fact that they can easily be settled, washed and the supernatant decanted. This method of extraction is undertaken on a routine basis by companies such as MicroBio Ltd. and Biosys for the mass production of entomopathogenic nematodes. Wilkenfeld et al., (1984) described the use of kimwipes placed onto the surface of the substrate which collects the nematodes, however this process of collecting nematodes would not be

feasible on a larger scale. The culture of nematodes is also in a monoxenic environment which is important in preventing the presence of contaminating organisms. This is important when introducing nematode cultures into larval shrimp growth tanks which are sensitive to contaminating organisms. Also the use of a liquid culture allows the addition of beneficial additives such as lipids and vitamins allowing also uniform enrichment.

Future work

The above research has demonstrated that *P. redivivus* can be cultured successfully in a 10L fermenter using a defined medium. Upscaling the culture to 100L and 1000L fermenter would be necessary to demonstrate that production is possible on this scale.

Investigation into an alternative to the egg component in the medium would be helpful. This would be beneficial on a large scale culture in 100L and 1000L fermenters as handling large amounts of raw egg can be awkward and therefore and alternative dry component such as dried egg would be easier to use.

CHAPTER TWO
LIPID ENRICHMENT OF NEMATODES

2. LIPID ENRICHMENT OF NEMATODES

INTRODUCTION

The absolute nutritional requirements of penaeid larvae are not known however relatively high levels of protein have been found to be important in the growth of early postlarval stages of *Penaeus stylirostris* and *P. californiensis* suggesting that larval protein requirement may be equal to or greater than 44% (Biedenbach et al., 1989). However a study by Kurmaly et al., (1989) showed that the differences of growth rates and survival of larvae of *P. monodon* fed on a variety of diets was not related to the general composition of the diets. An effective diet must be captured, ingested, assimilated and provide appropriate metabolites. (Kurmaly et al., 1989). Table 1 shows the overall nutritional composition of live and artifical feeds that have been successfully used to raise the larvae of *P. monodon* through to postlarvae. Tables 2 and 3 show the breakdown of fatty acids in live and artifical diets.

The lower production of the larvae reared on *D. tertiolecta* appears to reflect the lack of essential fatty acids eicosapentaenoic acid (20:5ω-3) and docosahexaenoic acid (22:6ω-3). The fatty acid requirement and the nature of fatty acid metabolism in penaeid larvae are not fully understood (Biendenbach et al., 1989). Ward et al., (1979) determined that the major fatty acids of the egg and nauplius of *P. setiferus* which remains the major fatty acids in the postlarval stage were 16:0, 18:0, 18:1ω-9, 20:4ω-6, and 20:5ω-6 and postulated that these fatty acids appear to be required in larger quantities than other fatty acids. Other studies have provided strong evidence that highly unsaturated fatty acids especially 20:5ω-3 and 22:6ω-3 play an important role in penaeid larval nutrition and may be required in larval diets (Jones et al., 1979a).

Table 1. Percenatge dry weight biochemical composition and physical characteristics of the diets fed to *P. monodon* larvae. After Kurmaly et al., (1989).

Diet	protein %	carbo-hydrate %	lipid %	Ash %	Size range (μm)	mean volume (μm^3)
Algal						
Tetraselmis chuii	48.8	24.7	4.3	22.2	10-19	430.44
Dunaliella tertiolecta	50.2	22.8	9.0	18.0	5-12	245.0
Rhodomonas baltica	52.0	33.7	4.3	10.0	6-10	125.6
Skeletonema costatum	33.3	22.6	8.1	36.0	8-16	114.4
Artificial						
Topal	40.0	35.0	15.0	6.0	7	175.5
Microencapsulated						
RDX10	57.0	14.0	15.0	14.0	1-10	268.0
X13D	54.0	17.0	13.4	15.6	1-10	212.1
RDX24	56.0	7.5	23.5	13.0	1-10	222.3
CAR005	49.0	27.0	13.0	11.0	1-10	229.7
CD435	51.0	17.8	18.2	13.0	40-90	33493.3

Table 2. Percentage fatty methyl ester composition of live diets as food for *P. monodon* larvae. After Kurmaly et al., (1989).

Sample	*T. chuii*	*T. suecica*	*R. baltica*	*S.costatum*	*D. tertiolecta*	*Artemia*
12:0						
14:0	4.0	1.4	4.9	19.7		2.0
15:0				0.5		0.5
16:0	26.0	29.1	11.0	10.7	18.0	13.2
16:1ω-9	0.7	0.9	0.5			0.3
16:1ω-7	0.6	0.6	1.3	32.6	1.7	21.0
16:1ω-5						
16:2ω-6						0.3
16:3ω-6						0.8
18:0	2.0	1.4	2.3	1.0	6.0	3.5
18:1ω-9,ω-11	11.5	10.5	3.9	1.7	6.6	33.0
18:1ω-7	4.3	3.0	3.2	1.2	2.7	
18:2ω-6	11.5	16.9	14.2	1.1	9.3	3.5
18:3ω-6	2.6	1.1	2.7	0.2	4.0	0.2
18:3ω-3	23.1	14.9	16.5	0.9	47.0	8.0
18:4ω-3	9.5	14.3	17.3	4.4	2.5	1.5
20:0						0.2
20:1ω-9.ω-11	0.7	0.6	0.4		1.8	
20:3ω-3						0.5
20:4ω-6	0.4	0.6	3.0	0.2		
20:5ω-3	3.4	4.6	11.6	22.9		9.5
22:1ω-11			0.2		1.2	0.5
22:5ω-3			0.1			
22:6ω-3			7.6	2.9		0.7
MUFA	18.2	15.62	9.07	35.5	14.0	64.41
PUFA	50.5	52.38	72.69	32.6	63.6	24.45
ω-3/ω-6	2.42	1.82	2.68	20.73	3.51	5.42

Table 3. Percentage fatty acid methyl ester composition of diets used as food for *P. monodon* larvae. After Kurmaly (1989).

Sample	*Topal*	*RDX10*	*X13D*	*RDX24*	*CAR005*	*CD435*
12:0					1.1	0.3
14:0		6.3	5.6	3.6	6.2	3.6
15:0						0.5
16:0	14.7	21.2	20.5	18.8	20.3	17.3
16:1ω-9	7.3	0.2	0.2	0.2	0.2	0.2
16:1ω-7	11.9	7.1	7.3	6.5	7.2	7.7
16:1ω-5						
16:2ω-6						0.3
16:3ω-6						0.8
18:0	2.3	4.0	4.3	3.9	3.7	4.8
18:1ω-9,ω-11	15.4	11.4	11.6	12.0	11.3	17.2
18:1ω-7	3.9	3.3	3.3	3.2	3.1	2.6
18:2ω-6	2.9	11.1	11.5	14.3	13.5	9.3
18:3ω-6						
18:3ω-3	1.2	2.1	2.0	2.4	2.2	1.0
18:4ω-3	2.8	2.0	2.1	2.0	1.9	1.9
20:0						
20:1ω-9.ω-11	4.1	2.4	2.0	2.2	1.9	9.0
20:3ω-3						
20:4ω-6		0.8	0.8	0.8	0.8	1.6
20:5ω-3	11.2	13.8	14.9	15.7	13.8	6.5
22:1ω-11	6.2	2.6	1.9	1.9	1.7	11.1
22:5ω-3	1.5	2.0	2.2	2.2	1.9	0.7
22:6ω-3	5.5	9.9	9.9	10.4	9.3	5.9
MUFA	57.98	27.0	26.3	26.0	25.31	57.02
PUFA	25.08	41.8	43.4	47.8	43.47	36.98
ω-3/ω-6	7.62	2.50	2.53	2.1	2.0	1.47

As well as an essential requirement for lipids crustacea have a large carbohydrate demand for synthesis of chitin. Large amounts of chitin are lost during each moult which is a major drain on the carbohydrate pool. The greater part of carbohydrate obtained from the diet is likely to be utilized for oxidative metabolism and chitin synthesis (Dall, 1967).

Previous studies using free living nematodes such as *Panagrellus sp* have shown that they could be used as a successful alternative live feed for *Artemia* (Plagman, 1939; Nikolsky,1963; Thomson, 1963, 1966; Gerlach and Schrage, 1969; Vallet et al, 1970; Opuszynsk, 1977, 1979; Rychly, 1977; Kahan et al,1975, 1979, 1980, 1983, 1984; Hofsten et al, 1983; Wilkenfeld et al, 1984; Biedenbach, 1989; Rottman et al, 1991).

Biedenbach et al., (1989) undertook a biochemical analysis (Table 4) and revealed that a significantly lower level of protein was present in nematode tissue (48%) compared to *Artemia* tissue (58%). Artemia cysts (Biomarine brand, China Strain, cysts size 250u, lot 692415) were hatched in aerated, artificial seawater at a salinity of 3ppt and temperature of 28 C. Nematodes however had a significantly higher carbohydrate level, which was three times that of *Artemia* and lipid levels were not significantly different between the two sources. Fatty acid analysis revealed that *Artemia* contained a greater percentage of Myristic (14:0), palmitic (16:0), palmitoleic (16:1ω-7), linolenic (18:3ω-3), arachidic (20:0) and eicosapentaenoic (20:5ω-3), while nematodes contained a greater percentage of steric(18:0), oleic (18:1ω-9), linoleic (18:2ω-6), nonadecyclic (19:0), heneicosanoic (21:0), arachidonic (20:4ω-6) and docosahexaenoic (22:6ω-3).

Fontaine and others (unpublished data) used several procedures for mass culture of *P. redivivus.* Their studies suggested that the media on which nematodes are cultured has an influence on growth characteristics of the nematodes (Radwin and Rouse, 1990). Rouse et al., (1992) was able to show that the ω-3 highly unsaturated fatty acids (HUFA) especially 20:5ω-3 and 22:6ω-3 could be increased by growing the nematodes on a medium containing wheat flour and fish oil. It was shown that these lipid enriched nematodes had a similar fatty acid profile to *Artemia* (Table 5).

Table 4. Fatty acid profiles of 1st and 2nd instar *Artemia* and 5 day old cultures of *Panagrellus redivivus*. After Biedenbach et al., (1989).

Fatty acid	*Artemia*	Nematodes (%)
14:0	6.261	0.549
16:0	9.902	8.538
16:1n-7	12.964	2.708
17:0	0.464	1.376
18:0	2.529	5.341
18:1n-9	18.145	19.144
18:2n-6	3.969	36.104
18:3n-3	3.031	1.729
19:0	0.721	1.111
20:0	1.978	0.826
20:2n-6	0.358	3.477
20:4n-6	1.504	4.534
20:5n-3	7.219	2.828
21:0	5.190	4.134
22:6n-3	0.078	0.195
Unidentified	25.683	7.406

It can be concluded from the work by Rouse et al., (1992) that by controlling the culture medium of the nematodes a more nutritionally constant live food can be produced.

By using twice the amounts of animal fats (beef fat which is high in saturated fatty acids) in solid culture, the levels of saturated fatty acids in steinernematid nematodes can be increased (Bedding, 1981, 1984). Most nematodes have a limited ability to synthesise lipids de novo and rely heavily on the host environment for essential nutrients (Chappell, 1979).

The objective of the following experiments were to manipulate the nutritional profile of the nematodes by increasing the levels of 22:6ω-3 and 20:5ω-3 which as previously discussed are important in the nutrition of the larval fish and shrimp species. Initial lipid analysis was undertaken on nematodes already in culture at the Littlehampton laboratory (AGC MicroBio Ltd.); *S.feltiae H.megidis*, *P.hermaphrodita*, *P. redivivus* and *C. elegans* and compared to lipid analysis undertaken by (Biedenbach et al, 1989).

A further objective was to explore the growth of *P. redivivus* on a variety of fish oils. Analysis was undertaken on the nematodes grown on the different fish oils to compare the levels of EFAs

Table 5. Percentage of fatty acids (wt.) in total lipids extracted from nematodes and two strains of *Artemia* (from Rouse et al., 1992)

Fatty acid	Nematodes grown on wheat flour and fish oil	*Artemia* (GSL) From Webster (1989)	*Artemia* (SFB) From Webster (1989)
12:0	0.2	0.26	0.08
14:0	4.67	0.78	1.24
14:1(ω-5)	1.52	0.98	0.36
16:0	12.89	14.12	11.11
16:(9ω-7)	10.46	20.52	3.34
17:0	0.42	1.11	1.67
18:0	4.7	7.51	4.07
18:1(ω-7)	11.28	8.78	7.70
18:2(ω-6)	9.91	8.23	4.78
18:3(ω-3)	9.28	28.19	3.76
20:0	0.23	0.63	0.6
20:1(ω-9)	1.02	0.58	0.56
20:3(ω-3)	0.44	0.27	0.34
20:4(ω-6)	4.64	1.72	2.15
20:5(ω-3)	7.35	1.19	9.32
22:0	0.47	0.27	0.04
22:1(ω-9)	1.52	0.12	0.10
22:2(ω-6)	0.78	0.36	0.12
22:4(ω-6)	0.08	0.01	0.01
22:5(ω-3)	0.11		
22:6(ω-3)	3.25		
%(ω-3)	20.41	13.44	29.68
%Saturates	23.56	22.13	22.54

content in the nematodes. The oil that provided optimum growth and enrichment to the nematodes could then be used in larger scale culture. The effect of levels of the fish oil in the medium were also explored.

MATERIALS AND METHODS

The extraction, axenization and general culture of *S. feltiae*, *H. megidis, P. hermaphrodita, C. elegans* and *P. redivivus* is discussed in the 'materials and methods' in chapter 1.

Experimental conditions;

Bacteria: *Xenorhabdus nematophilus/ Escherichia coli* (*P. redivivus* and *C. elegans*)

Medium: 10% kidney, 3.5% oil, 1% yeast extract.

Flasks: 250ml baffled flasks containing 50ml of medium

Inoculation level : *P. redivivus*, 2000ml^{-1}; *C. elegans*, 500ml^{-1}; *P. hermaphrodita*, 1500ml^{-1}; *S. feltiae* and *H. megidis* 3000ml^{-1}.

Culture temperature: 22^{0}C(15^{0}C for *P. hermaphrodita*).

pH; all flasks were calibrated to pH7.

Preparation of nematode samples

Analysis of samples is undertaken in two stages: 1) all lipids are extracted from the sample, 2) fatty acids are removed from the lipid sample and converted into a chemical form suitable for analysis by the GLC. Extraction of lipids from freeze dried samples is undertaken using a mixture of chloroform/methanol (2 parts chloroform to 1 part methanol) containing a little BHT (2,6 Di-tert-butyl-p-cresol) which acts as an antioxidant, approximately 1 crystal to 500ml of solvent. Only glass vessels were used as the solvent extracts plasticisers from plastic.

Approximately 10ml of solvent was added to the sample and homogenised The sample was left in a cold room for complete extraction for about 20 min. The extract was then filtered (no.4 filter paper) into a stoppered measuring cylinder containing 2 mls of 0.017% aqueous magnesium chloride and briefly agitated. The sample was then decanted into a centrifuge tube and covered with aluminium foil. The sample was then centrifuged at 2000 rpm for 5 min. Two separate phases occur, the upper phase was removed by aspiration (a pasteur pipette is connected to a vacuum pump (worked from a water tap) by a piece of vacuum tubing. The lower phase was then washed with Folch Upper phase (chloroform:methanol:water 3:48:47). A little Folch Upper phase was poured into the tube so that it formed a layer on top of the lower phase.

All the lipids were now present in the lower phase and the solvent was then removed using a Rotary Evaporator. This applies a vacuum to the solvent so that only gentle heat was needed to evaporate it. The sample was then poured through a filter paper containing a small amount of anhydrous sodium sulphate (removed any remaining water) and washed through with a little

chloroform. The flask was then connected to a vapour duct and lowered into a water bath set at 30^0C. The vacuum gauge registered about 530nm of mercury and solvent was seen condensing on the cooling coils. When there was no more condensate formation the samples were dry.

The lipid was then dissolved in chloroform (approx 1ml) and transferred using a pasteur pipette to a preweighed vial in a heating block. The chloroform was then evaporated with a stream of oxygen free nitrogen (OFN). As soon as the sample was dry it was weighed. The lipid was then redissolved in 1ml of chloroform and 2ml of methylating reagents (14% boron triflouride in methanol). The sample vial was then placed in a heating block at 100^0C for 1 h.

When the vials had cooled sufficiently the contents were poured into 14 ml glass vials and 3ml of water and 6 ml of pentane were added. A Teflon lined cap was screwed on, agitated and the contents allowed to settle. The upper pentane layer was transferred to another vial and the procedure was repeated a second time with 6ml of pentane. The pentane was evaporated with OFN and the fatty acid methyl esters were dissolved in hexane. The sample contained fatty acid methyl esters which were ready to be analysed by GLC. At this point an internal standard was added to the machine to quantitate the analysis (Folch et al., 1957; Morrison and Smith, 1964).

Experiment 1

Lipid analyses of *P. redivivus*, *S. feltiae, H. megidis* and *P. hermaphrodita*.

All nematodes were cultured and extracted from flasks as described in chapter 1 'Materials and Methods' in a meduim containing 10% wet weight kidney. This was to ensure that nematodes were grown in similar conditions on a 'fail safe' medium with their respective associated bacteria.

The nematodes *P. redivivus*, *S. feltiae, H. megidis* and *P. hermaphrodita*, were analysed for their lipid and protein content to see if they contained adequate quantities of the essential fatty acids.

Experiment 2

The manipulation of the fatty acid profile of the nematode *S. feltiae*.

Experiment 2 attempts to manipulate the lipid profile of *S. feltiae* by increasing the levels of 20:6ω-3 and 20:5ω-3 by the addition of capelin oil (a fish oil obtained from Sea maid UK.) to the growth medium of the nematodes so that they are elevated to a level equal to that of *Artemia.* The components for the medium used were 10% kidney, 3.5% corn oil and 1% yeast extract (wet weight). The corn oil component was manipulated by altering the percentage of corn oil used with capelin oil high in the essential fatty acids 20:5ω-3 and 22:6ω-3 (Table 6).

Table 6. Percentage of corn oil and capelin used in 5 treatments

Treatment	Corn/capelin oil %	Kidney %	Yeast extract %
1	100/0	10	1
2	75/25	10	1
3	50/50	10	1
4	25/75	10	1
5	0/100	10	1

The overall oil percentage in the medium remained the same. Nematodes were extracted from 50ml flasks by using the method described in chapter 1. The nematode slurry was allowed to settle and the supernatant was decanted off. The nematodes were then filtered to remove any excess water and the resulting nematode paste was placed into 5ml vials and frozen. Frozen samples were then freeze dried. Freeze dried samples were then ready for lipid analysis using a gas liquid phase chromatography technique.

Experiment 3

The manipulation of the fatty acid profile of the nematode *H. megidis.*

The experiment attempts to manipulate the lipid profile of *H. megidis* by increasing the levels of 20:6ω-3 and 20:5ω-3 by the addition of fish oil to the growth medium of the nematodes so that they are elevated to a level equal to that of *Artemia.* Identical culture methods in experiment

2 were used in experiment 3.

Experiment 4

The manipulation of the fatty acid profile of the nematode *P. redivivus*.

Attempts were made to increase the levels of essential fatty acids in the nematode *P. redivivus* by the addition of capelin oil to the growth medium. It was established from the experiments 3 and 4 that the levels of 20:5ω-3 and 22:6ω-3 could be elevated in *S. feltiae* and *H. megidis* by the addition of capelin oil. Identical culture methods in experiment 2 were used in experiment 4.

Experiment 5

***P. redivivus* grown on a variety of fish oils.**

This experiment explores the variety of fish oils available containing high levels of the EFAs and their addition to the growth medium of this nematode. The growth of the nematode was observed for each fish oil. Lipid analysis was undertaken on nematodes grown on Marilla oil, cod liver oil and capelin oil. Identical culture methods in experiment 2 were used in experiment.

RESULTS

Experiment 1

Table 7. shows the total protein and lipid content of *P. redivivus*, *S. feltiae, H. megidis* and *P. hermaphrodita*. All data is presented as % of dry weight.

The results in Table 7 show that the selection criteria of protein 40-50% and lipid 15-20% of the dry weight is met in all these nematodes. Table 7a indicates the values expressed as % of total lipid. The remainder of each sample contained almost equal proportions of free fatty acids, sterol esters, partial acylglycerols and sterols. Table 7b indicates significant proportions 18:1ω-9 and

Table 7 Total protein and lipid content of *S. feltiae*, *H. megidis*, *P. hermaphrodita* and *P.redivivus.*

Sample	Protein	Lipid
Steinernema feltiae	54.5	24.4
Heterorhabditis megidis	53.5	24.1
Phasmarhbditis hermaphrodita	55	16.2
Panagrellus redivivus	48.3	17.3

Table 7a Triacylglycerols and Polar lipids of *S. feltiae*, *H. megidis* and *P. hermaphrodita.*

Sample	Triacylglycerols	Polar lipids
Steinernema feltiae	69.5	5.1
Heterorhabditis megidis	58.5	4.5
Phasmarhbditis hermaphrodita	51.7	7.6

18:2ω-3 which are high and due to possible storage utilisation by the nematodes (Selvan and Jenkins, 1993). Whilst 22:6ω-3 is very low or absent in the nematodes, the 20:5ω-3 level is reasonable and is above the required selection value of 1.5% of the total value of the lipid in *S. feltiae,* and *P. hermaphrodita.* However *P. redivivus* has a total value of 1.42% for 20:5ω-3 and is below the required value.

Table 7b Fatty acid profile of *S. feltiae*, *H. megidis*, *P. hermaphrodita* and *P.redivivus.*

Fatty acid	*S. feltiae*	*H. megidis*	*P. hermaphrodita*	*P.redivivus*
14:0	0.2	0.4	0.2	0.27
iso - 15:0	1	1.8	0.5	
ante - 15:0	-	1.4	0.5	
15:0	-	-	-	
iso 16:0	-	1	-	
16:0	3.9	2.2	3	6.77
16:1(n-7)	0.2	0.6	0.5	0.88
16:1(n-5)	0.1	2.6	0.4	
iso - 17:0	0.2	2.4	0.8	
ante - 17:0	0.2	3.5	0.4	
17:0	0.4	0.5	-	
iso - 18:0	1.5	0.6	0.8	
18:0	3.7	3.3	6.1	5.11
18:1 (n-9)	19.9	22.6	21.5	18.61
18:1 (n-7)	-	4.6	3.9	3.9
18:2 (n-6)	55.1	41	39.8	24.65
18:3 (n-6)	0.3	1.3	0.7	
18:3 (n-3)	0.8	0.9	0.6	0.56
20:0	0.5	0.2	0.3	
20:1 (n-9)	2.1	0.2	0.5	0.91
20:2 (n-6)	4	1	4	1.2
20:3 (n-6)	0.8	1.4	1.5	3.29
20:4 (n-6)	0.7	2.2	4	8.59
20:3 (n-3)	0.3	-	0.2	
20:4 (n-3)	0.6	-	0.6	
20:5 (n-3)	**2.2**	**0.5**	**6.3**	**1.42**
22:0	0.1	-	0.3	
22:1 (n-9)	0.1	-	-	
22:6	-	-	**trace**	**-**
Unknowns	1.1	3.8	2.6	

Experiment 2.

The manipulation of the fatty acid profile of the nematode *S. feltiae*

This experiment was an attempt to manipulate the fatty acid profile of *Steinernema feltiae* by adding capelin oil to the lipid component of the medium in varying percentages.

Table 8 shows that levels of protein and lipid comparable to that in *Artemia*, the lowest protein content (46.5%) and highest lipid content (29.7%) were obtained from nematodes grown in a medium containing 25% corn oil: 75% capelin. Table 8a and 8c shows that with increasing levels of capelin oil in the oil component of the diet, 20:5ω-3 and 22:6ω-3 increases in the nematode. Levels of 18:2ω-6 decrease but 18:1ω-9 remains constant. There is also the presence of 17:0 which is indicative of the presence of a bacterium. When the composition of capelin oil and cod liver oil are compared in Table 8b it can be seen that 18:2ω-6 and 18:1ω-9 are very high in the corn oil and very low in the capelin oil. This is reflected in the nematodes grown on a medium containing these oils. Nematodes grown on corn oil reflect a similar fatty acid profile to the corn oil and those grown on capelin also show a similar fatty acid profile to the capelin oil. 22:6ω-3 and 20:5ω-3 are absent in the corn oil but present in very high proportions in the capelin oil along with other fatty acids of the 22:0 series. High levels of monosaturates and saturates are present in the capelin oil. Figure 1 shows that with increasing capelin oil the essential fatty acids 20:5ω-3 and 22;6ω-3 also increase.

Table 8 Protein and lipid contents of *S. feltiae* (dry weight) grown in different ratios of corn and capelin oils.

Total %	Corn oil 100%	Corn oil 75%	Corn oil 50%	Capelin oil 75%
Protein	50	55	53.6	46.5
Lipid	26.6	22.7	25.7	29.7

Table 8a Fatty acid composition (dry weight %) of nematodes

Fatty acid	corn oil 100%	corn oil 75%	corn oil 50%	corn oil 25%
14:0	0.2	0.6	1	1.4
14:1	0.9	1.1	1	1
15:0	-	-	0.2	0.1
16:0	5.1	4.5	5.3	5.3
16:1(ω-7)	0.3	0.4	0.8	0.9
16:1 (ω-5)	0.3	0.7	1.2	1.2
i-17:0	0.3	0.4	0.4	0.4
a-17:0	0.1	0.1	0.1	0.1
16:2	-	-	-	-
16:3	-	-	-	-
17:0	0.2	0.2	0.3	0.3
i -18:0	2.2	2.3	2.2	2.2
18:0	5.9	5.2	5.4	4.7
18:1 (ω-9)	19.3	17.9	17.5	16.8
18:1 (ω-7)	1.2	1.6	2.9	2.4
18:2 (ω-6)	47.4	38.3	29.7	21.3
18:3(ω-6)	0.9	0.4	0.4	0.8
18:3(ω-3)	0.9	0.4	0.4	0.8
18:4(ω-3)	-	0.6	0.8	1.3
20:0	0.6	0.5	0.6	0.5
20:1 (ω-11/9)	2.1	4.9	7.2	8.8
20:1(ω-7)	0.1	0.1	0.3	0.2
20:2(ω-6)	4	3.5	2.6	1.8
20:3 (ω-6)	0.9	1.2	0.8	0.9
20:3(ωn-3)	0.5	0.4	0.2	0.2
20:4(ω-6)	0.7	1	0.9	0.9
20:4 (ω-3)	0.9	1.9	2.5	3.5
20:5 (ω-3)	**3.0**	**5.8**	**7.0**	**9.2**
22:0	0.1	0.2	0.3	0.3
22:1 (ω-11)	0.1	1.8	3.5	4.5
22:1(ω-9)	-	0.2	0.4	0.4
22:2	-	-	-	0.2
22:5 (ω-3)	-	0.2	-	0.6
22:6(ω-3)	-	**1.3**	**1.7**	**2.8**
24:1(ω-9)	-	-	-	0.7
Total sats.	14.7	14	15.8	15.3
Total monos	24.3	28.7	34.8	36.4
Total PUFA	59.2	55	46.9	44
Unknowns	1.8	2.3	2.5	4.3

Table 8b. Fatty acid composition of corn oil and capelin oil

Fatty acid	Corn oil 1	Corn oil 2	Capelin oil 1	Capelin oil 2
14:0	-	-	7.8	7.8
14:1	-	-	0.2	0.3
15:0	0.1	-	0.4	0.4
16:0	10.3	10.3	13.3	13.7
16:1 (ω-7)	0.1	0.1	5.7	5.7
16:2	-	-	1.1	0.8
16:3	-	-	0.4	0.3
16:4	-	-	0.7	0.5
18:0	1.9	2	1.7	1.6
18:1 (ω-9)	28.9	29.3	8.4	8.6
18:1 (ω-7)	1.1	1	1.8	1.7
18:2 (ω-6)	55.2	54.9	2.1	2
18:3 (ω-3)	1.4	1.3	1.9	1.9
18:4(ω-3)	-	-	4.8	5
20:0	0.5	0.5	0.3	0.3
20:1 (ω-9)	0.4	0.4	12.6	12.7
20:2(ω-6)	-	-	0.2	0.3
20:4 (ω-6)	-	-	0.3	0.3
20:4(ω-3)	-	-	1	0.8
20:5(ω-3)	-	-	6.6	6.8
22:1 (ω-11/9)	-	-	18.4	18.3
22.2	-	-	0.4	0.4
22.4	-	-	-	-
22:5(ω-3)	-	-	0.8	0.8
22:6(ω-3)	-	-	8.1	8
24.1 (ω-9)	-	-	0.9	0.7
Total sats.	12.8	12.8	21.8	23.8
Total monos.	30.5	30.8	48	48
Total PUFA	56.6	56.2	30.1	27.9

Table 8c. Percentage dry weight of EFAs found in *P. redivivus* when grown on a medium containig varying levels of capelin oil/corn oil.

Fatty acid	100% capelin	25% corn oil	50% corn oil	75% corn oil	100% corn oil
20:5ω-3	No survival	9.20	7.00	5.80	3.00
22:6ω-3	No survival	2.80	1.70	1.30	0.00

Experiment 3

The manipulation of the fatty acid profile of the nematode *H. megidis*.

The objective of this experiment was to increase the levels of the essential fatty acids 20:5ω-3 and 22:6ω-3 in the nematode *Heterorhabditis megidis* Table 9 shows that although replacement of corn oil with capelin oil does not effect total lipid levels, protein levels are reduced and below shrimp larvae requirements. However Table 9a demonstrates that a 25% capelin oil replacement is successful in elevating 20:5ω-3 and 22:6ω-3 levels to those necessary for larval shrimp.

Table 9 The percentage dry weight of protein and lipid present in the nematode *H .megidis* grown in varying amounts of capelin oil.

	% Capelin oil replacing corn oil				
	0%	25%	50%	75%	100%
Protein	33.6	22.0	20.0	16.0	29.9
Lipid	25.5	24.8	24.4	23.6	26.2

Table 9a. The percentage dry weight of the fatty acids 20:5ω-3 and 22:6ω-3 present in *H. megidis* when grown on varying levels of capelin oil.

	% Capelin oil replacing corn oil				
Fatty acid	0%	25%	50%	75%	100%
22:6ω-3	0	1.0	2.5	3.0	3.6
20:5ω-3	25.5	3.5	6.8	9.3	10.7

Experiment 4

The manipulation of the fatty acid profile of the nematode *P. redivivus*.

Using the above methods as in experiments 1, 2 and 3, the free living nematode *P.redivivus* was

grown on varying levels of capelin oil. The objective of this experiment was to investigate if the levels of the essential fatty acids 20:5ω-3 and 22:6ω-3 could be elevated by increasing the fish oil percentage in the oil component.

Table 10. Total lipid and protein present in *P. redivivus* fed varying levels of capelin oil

	% Capelin oil replacing corn oil				
	100	75	50	25	0
% Lipid	19.45	11.73	19.6	25.11	13.8
% Protein	28.27	31.96	27.17	30.5	27.15

Table 10a. Percentage dry weight of the EFAs 20:5ω-3 and 22:6ω-3 present in *P. redivivus* grown on varying levels of capelin oil

	% Capelin oil replacing corn oil				
Fatty acid	100	75	50	25	0
20:5ω-3	7.4	7.08	6.82	5.74	1.97
22:6ω-3	0.51	0.49	0.44	0.39	0.09

It can be seen from the results in Table 10 that by increasing the percentage of capelin oil in the oil component of the medium the total dry weight percentage of lipid content increases. However the protein content declines with increasing capelin oil content. From Table 10a it is clear that there is an elevation of the EFAs; 20:5ω-3 and 22:6ω-3 with increasing capelin oil content (see Fig. 2). However there is little further increase in 22:6ω-3 above a 25% capelin oil level in the diet.

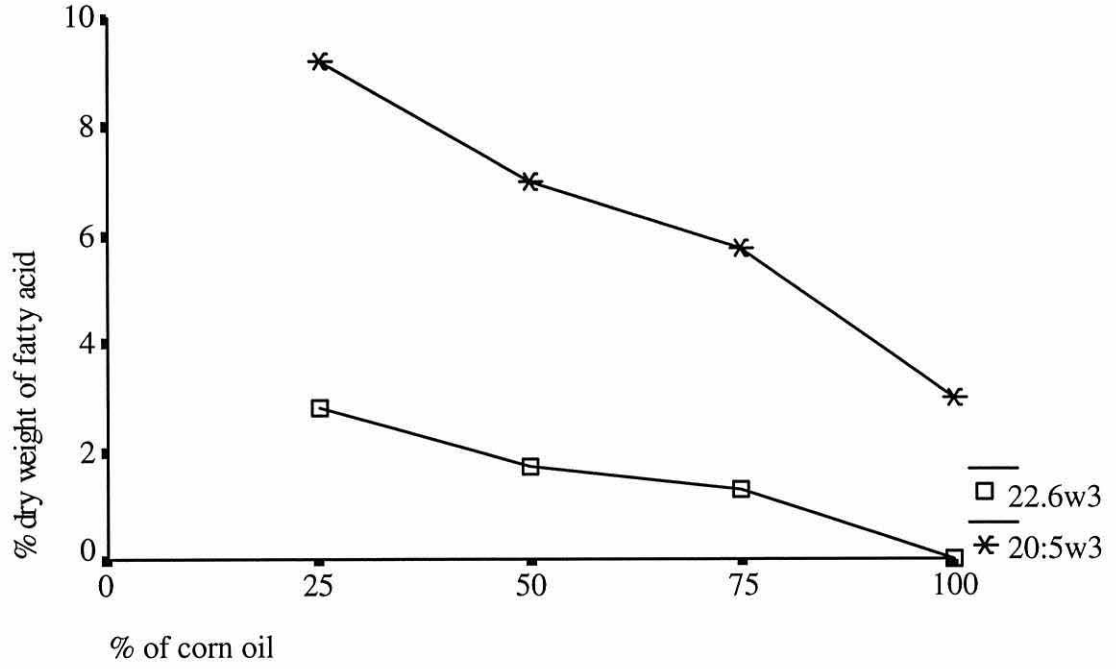


Figure 1. *S. feltiae* grown on a medium containing increasing percentages of capelin oil in the lipid component of the medium. The graph shows that with increasing levels of capelin oil the dry weight percentages of the fatty acids; 20:5ω-3 and 22:6ω-3 also increase in the nematodes.

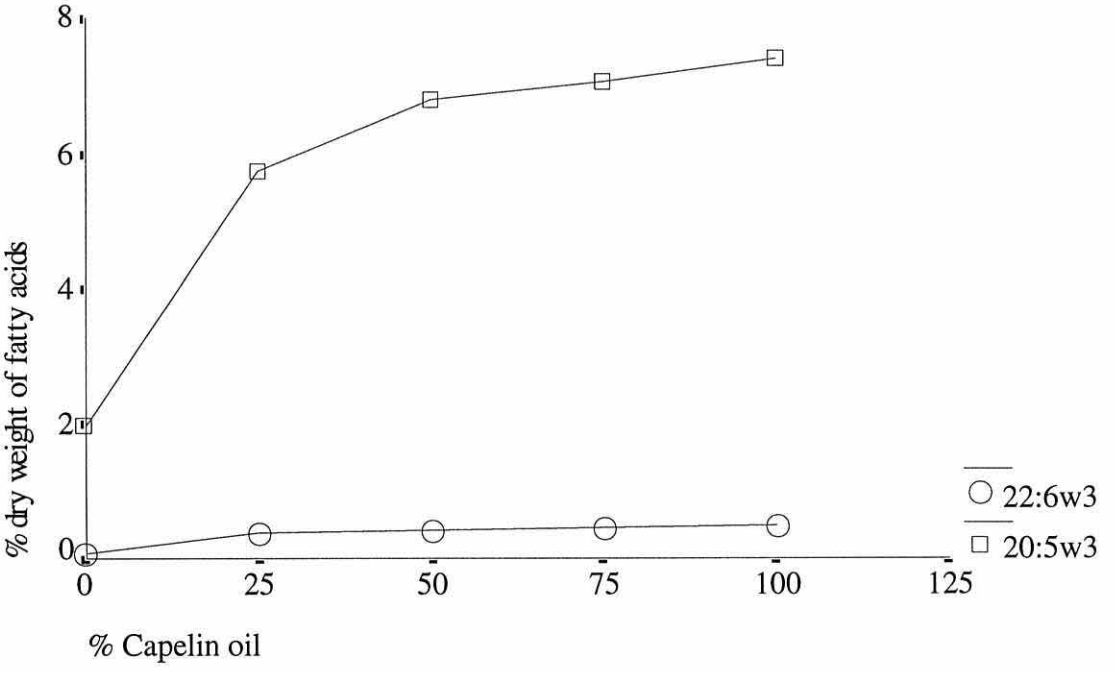


Figure 2. *P. redivivus* grown on a medium containing increasing percentages of capelin oil in the lipid component of the medium. The graph shows that with increasing levels of capelin oil the dry weight percentages of the fatty acids; 20:5ω-3 and 22:6ω-3 also increase in the nematodes.

Experiment 5.

***P. redivivus* grown on a variety of fish oils.**

Capelin oil

The results shown in Table 11 indicate that treatments containing 100% and 75% corn oil remain significantly different to other treatments and produce higher population numbers. The least effective treatments were treatments containing 100% and 75% capelin oil. The EFAS 20:5ω-3 and 22:6ω-3 also were shown to increase with increasing capelin oil percentage within the diet (Table 14) Fig.3 shows the growth of *P. redivivus* on capelin oil. Throughout the experiment the control containing 100% corn oil remains significantly different to other treatments at the 5 % level. However growth of the nematodes were comparable to treatments containing 25% and 50 % corn oil. After day 17 growth declines rapidly for treatments containing 75% and 100% capelin oil.

Table 11. *P. redivivus* mean population counts of 4 replicates grown on varying percentages of capelin oil replacing corn oil.

Day	0% cape	25% cape	50% cape	75% cape	100% cape*	Significant
0	2000.00	2000.00	2000.00	2000.00	2000.00	*
3	5025.00	13450.00	13700.00	11800.00	13900.00	*
7	36062.50	41687.50	39562.00	3387.00	33062.50	*
12	153500.00	162000.00	140000.00	115000.00	112000.00	*
17	151250.00	172000.00	154500.00	143000.00	125500.00	*
24	183500.00	194750.00	173500.00	132500.00	81000.00	*
27	113250.00	178500.00	182000.00	220000.00	139000.00	*

Cod liver oil

Growth was adequate on all levels of cod liver oil however the significant difference at the 5% level towards the end of the experiment of the control treatment (100% corn oil) compared to the other treatments was due to a slow growing contaminating bacterium. The presence of this bacterium increased the growth of the nematodes and gave a false result. Nematodes continued to reproduce whereas normally growth would decline. The treatment containing 100% codliver

oil produced significantly lower populations at the 5% level compared to all the other treatments, however it maintained good growth and development through out the experiment.

Table 12. *P. redivivus* mean population counts of 4 replicates grown on varying percentages of codliver oil

Day	cod 100%	cod 75%	cod 50%	cod25%	corn 100%	Significant
0	2000	2000	2000	2000	2000	*
4	81500	69100	71800	63400	72700	*
10	128000	121000	110000	104000	127000	*
14	132000	107000	85000	115000	121000	*
18	123000	119000	88000	79000	81000	*
22	162000	126000	102000	94000	99000	*

Marilla oil

The treatment (Table 13) containing 50%Marilla oil was significantly different at the 5% level from all the other treatments producing the poorest growth. However the highest and lowest means throughout the experiment were not significantly different from all the other treatments. At day 22 the control produced the highest maximum population of 139 275 nematodes ml^{-1} of all the treatments.

Table 13. *P. redivivus* mean population counts of 4 replicates grown on varying percentages of marilla oil replacing corn oil.

Day	100% corn	25% marilla	50% marilla	75% marilla	100%marilla	Significant
0	2000.00	2000.00	2000.00	2000.00	2000.00	*
5	35000.00	27075.00	750.00	33225.00	32875.00	*
9	87500.00	81875.00	0.00	80500.00	76000.00	*
16	57968.75	54250.00	0.00	47375.00	39125.00	*
22	139275.00	87500.00	0.00	75000.00	70875.00	*

Table 14 . Percentage nutritional and fatty acid composition of the nematode *P. redivivus* grown on diets with different oil components; corn oil, capelin oil, marilla oil and cod liver oil.

Fatty acid	corn oil 100%	capelin oil 50%*	marilla oil 50%	cod liver oil 50%
Protein (%)	77.0	52.9	59.1	51.9
Lipid (%)	14.8	16.9	19.9	18.8
14:0	0.27	1.96	0.81	1.17
16:0	6.77	8.22	3.68	7.61
16:1(ω-9)	0.463	0.8	0.33	0.28
16:1 (ω-7)	0.88	2.7	2.92	2.84
18:0	5.11	2.78	2.51	3.4
18:1 (ω-9)	18.61	18.14	5.62	17.57
18:1 (ω-7)	3.9		8.54	4.42
18:2 (ω-6)	24.65	25.17	22.29	26.87
18:3(ω-3)	0.56	0.99	0.75	0.92
18:4(ω-3)	0.23	1.15	0.85	0.65
20:0		0.2	0.25	0.19
20:1 (ω-9)	0.91	4.7	4.69	4.81
20:2(ω-6)	1.2	0.63	1.1	0.68
20:3 (ω-6)	3.29	0.68	1.82	0.85
20:4(ω-6)	8.59	1.54	2.58	1.87
20:4 (ω-3)		0.24	1.82	0.5
20:5 (ω-3)	**1.42**	**5.17**	**6.83**	**5.73**
22:1 (ω-11)		7.25	1.09	0.51
22:5 (ω-3)	-	0.41	1.41	0.62
22:6(ω-3)	-	**3.91**	**3.74**	**2.86**
Total sats.%	12.15	13.16	7.25	12.37
Total monos%	24.76	33.59	23.19	30.43
Total PUFA%	39.94	39.89	42.69	41.55
Sum %	76.85	86.64	73.13	84.35
ω-3	2.21	11.87	14.9	11.28
ω-6	37.73	28.02	27.79	30.27
ratioω-3/ω-6	17.07	2.36	1.87	2.68

* Different source from that analysed in Table 10.

From Table 14 it can be seen that corn oil produces the highest dry weight protein content of 77% compared to the other fish oils. For all treatments the total lipid content falls between the desired level of 15-20%, however nematodes grown on corn oil fell marginally below this figure at 14.8%. For the storage fatty acids (18 series) treatments were comparable in their content except for marilla oil only pocessing 5.62% (18:1ω-9).

For EFAs such as 20:5ω-3 corn oil contained 1.42% compared to 5.17 (capelin), 6.53 (marillla) and 5.73 (cod liver). The fatty acid 22:6ω-3 was absent in corn oil but present at levels of 3.91, 3.74 and 2.86 for capelin oil, marilla oil and codliver oil respectively. The ratio of ω-3/ω-6 is highest in corn oil at 17.07% compared to all other treatments.

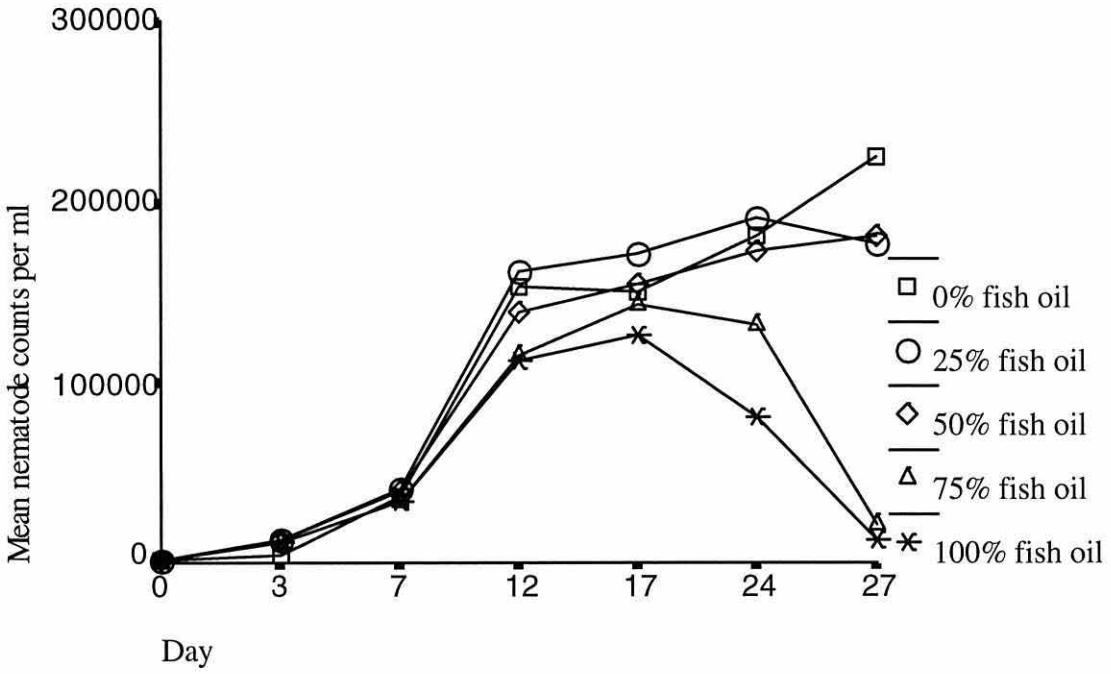


Figure 3. *P. redivivus* grown on varying levels of capelin oil as a replacement for corn oil. A mean of 4 replicates was assessed. Nematodes were then harvested and analysed for their fatty acid content.

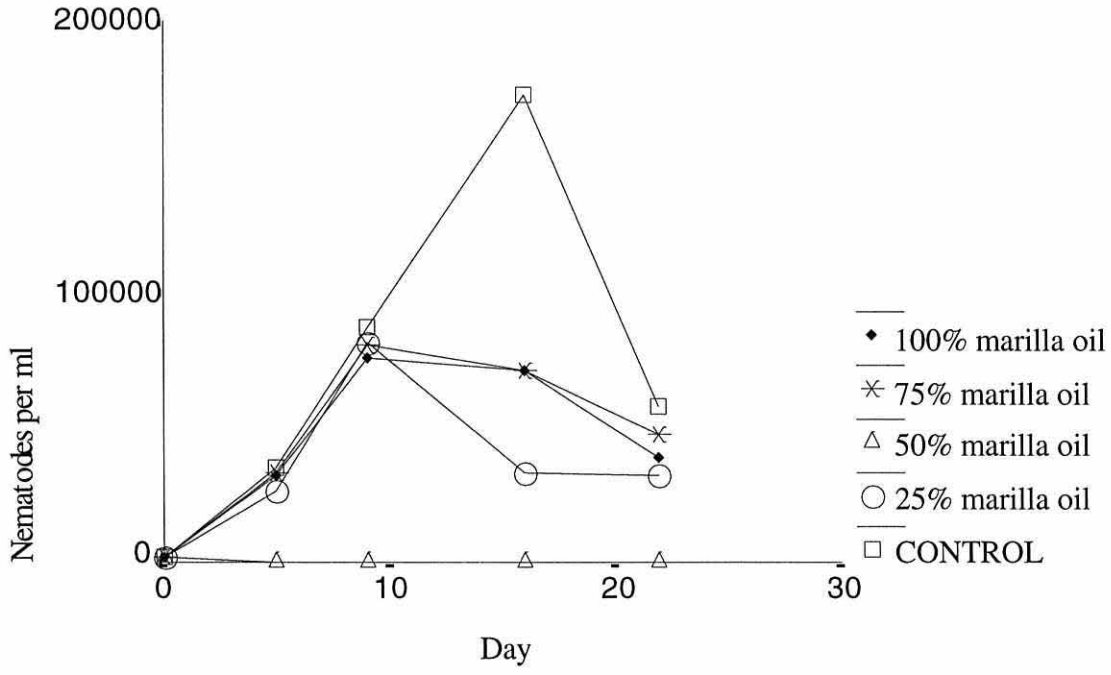


Figure 4. *P. redivivus* grown on varying levels of marilla oil as a replacement for corn oil. A mean of 4 replicates was assessed. Nematodes were then harvested and analysed for their fatty acid content.

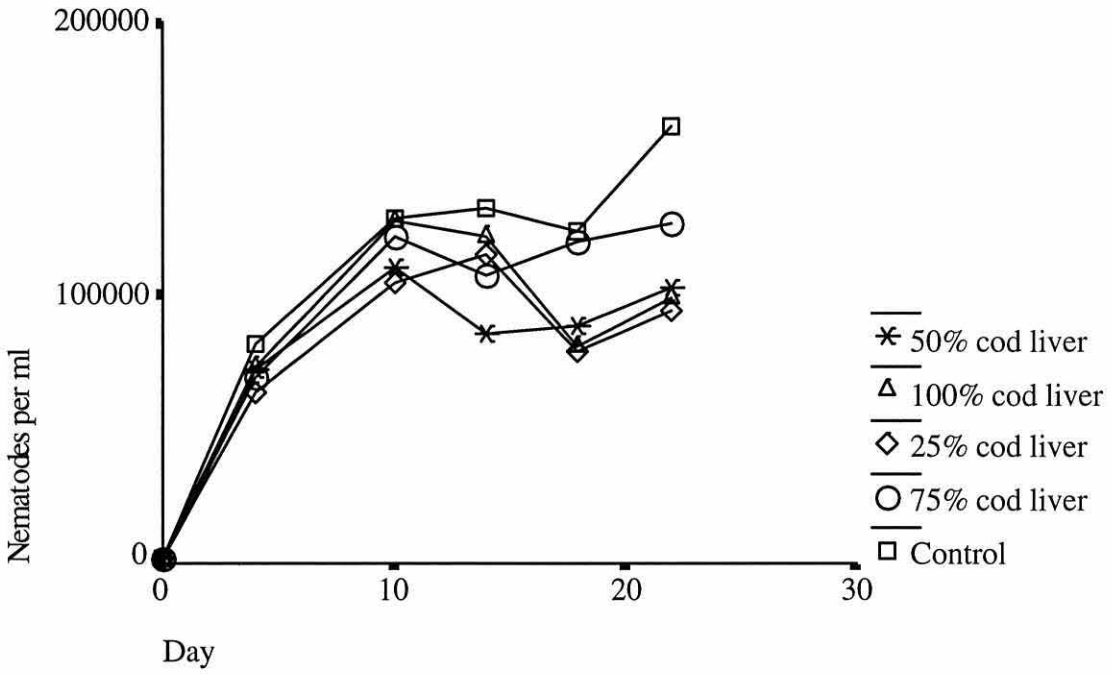


Figure 5. *P. redivivus* grown on varying levels of cod liver oil as a replacement for corn oil. A mean of 4 replicates was assessed. Nematodes were then harvested and analysed for their fatty acid content.

DISCUSSION.

S. feltiae, H. megidis, P. hermaphrodita and *P. redivivus.*

The analyses show that *S. feltiae, H. megidis P. redivivus* and *P.hermaphrodita* have a lipid and protein profile within the desired selection criteria as described earlier for protein 40-50% and lipid 15-20% expressed as a dry weight percentage and hence should be suitable for shrimp larvae nutrition.

The survival of these infective juveniles is dependant on the accumulation of energy reserves which are thought to be lipids (Van Grundy et al., 1987). In entomopathogenic nematodes the energy reserves are predominantly saturated fatty acids (Selvan et al., 1993). However these nematodes have to rely on their host and surrounding environment for the source of these lipids as they have limited capabilities of producing their own (Chappel, 1979). Lipids provide 60% of the energy reserves in infective juveniles (Selvan et al., 1993) and saturated fatty acids provide more energy than non saturated fatty acids (Stryer, 1988).

The results presented in Experiment 1 agree with Selvan et al, (1993) in that *S. feltiae* has more stearic acid (18:0) than oleic acid (18:1ω-9) but not as high as the 23-25% as stated. *P. hermaphrodita* has a much higher content of stearic acid in the range of 54-60%. These results however disagree with the results presented by Selvan (1993) who stated that *Steinernema sp.* contain higher levels of saturates than *Heterorhabditis sp* particularly *H .bacteriophora. Steinernema sp* have a better storage and survival than *Heterorhabditis sp*, the latter has a higher infectivity but is less able to survive environmental extremes. This may be a reflection on the differing amounts of fatty acids in the nematodes as saturates appear to be higher in *Steinernema sp.* (Klein 1990; Selvan et al., 1993). These results show that there is little difference in the overall saturate content in *Steinernema* and *Heterorhabditis.*

The docosahexaenoic (22:6ω-3) fatty acid is very low or absent in all species however eicosapentaenoic (20:5ω-3) is present at acceptable levels and is particularly high in *P. hermaphrodita* and *P. redivivus.* When the corn oil component of the medium was analysed it

was found to be devoid of both these fatty acids 22:6ω-3 and 20:5ω-3. This suggests that these nematodes to a certain extent can manufacture these fatty acids and *S feltiae* and *H. megidis* can manufacture 20:5ω-3. Evidence has been shown that 20:5ω-3 is present in high amounts in the L3 nematode larvae of *Brugia malayi* and was actively being biosynthesised. Eicopolyunsaturated fatty acids are known to be metabolised in mammalian cells into eicosanoids and are known to mediate a range of cellular responses (Liu and Weller, 1989).

The results also indicate the presence of the hexadecanoic (17:0) fatty acid. This usually indicates the presence of a bacterium which in the case of *S. feltiae* and *H. megidis* is due to the presence of an associated bacterium present in the gut in the infective juvenile stage.

The levels of the essential fatty acids (EFAs) 22:6ω-3 and 20:5ω-3 can be successfully increased in the nematodes *S. feltiae* and *H. megidis* when grown on a medium containing increased levels of capelin oil. Analysis of this oil has shown it to contain very high levels of these EFAs and the maximum level of capelin oil that could be incorporated into the corn oil component was 75% and 100% for *S. feltiae* and *H. megidis* respectively. The presence of these fatty acids was well in excess of 1.5% of the dry weight.

When capelin oil and corn oil were analysed and compared it was found that corn oil has over 50% of its total dry weight of octadecenoic acid 18:1ω-3 and very high levels of octadecadienoic 18:2ω-3. These fatty acids are thought to be important in nematode storage. Corn oil has a high percentage of its fatty acid components in the 18 carbon series, whereas capelin has a high percentage of fatty acid components in the 20 carbon range. This explains the difference in fatty acid composition of nematodes grown on varying levels capelin and corn oil as nematodes reflect the composition of the medium in which they are grown. However total protein content declines in *H. megidis* with increasing percentage of capelin oil and although this decrease in protein does not seem to be detrimental to the growth of the nematodes it may affect efficacy as a diet.

P. redivivus.

Total analysis of *P. redivivus* reveals the protein dry weight composition to be in the region of 45-50% and the lipid content to be in the region 15 -20%. *P redivivus* contains high proportions of 18:2ω-6 and 18:1ω-9. There is evidence to suggest that the nematodes can convert 18:1ω-3 to 18:2ω-6 and this to eicosadienoic acid 20:2 and to eicosenoic acid 20:1. This is possibly due to the nature of the bacterial food which they consume in their natural environment. The bacteria are high in c18s and c20s which constitute 80% of the total lipid present (Van Grundy, 1987). However variation in analyses may be observed due to the wide age distribution in cultures at the time of harvest.

20:5ω-3 is present in *P. redivivus* which suggests that it possesses the enzymatic pathway to manufacture this fatty acid as it is not present in the corn oil component of the medium. The results also demonstrate that by increasing the capelin oil component of the medium the levels of the EFAs are also elevated. The results further demonstrate that the nematode can grow on a variety of fish oils which possess a high percentage of the EFAs 22:6ω-3 and 20:5ω-3.

When the results obtained for *P. redivivus* are compared to *Artemia* (Table 15) it can be seen that nematodes grown on a medium containing fish oil such as capelin oil show a comparable lipid and protein profile to that of *Artemia*. However the findings are different to those obtained by Biedenbach et al., (1989) in that the overall lipid content is higher to that found in *Artemia*. Nematodes had elevated levels of myristic (14:0), palmitic (16:0), Palmitoleic (16:1ω-7), eicosapentaenoic (20:5ω-3) and highly elevated levels of docosahexenoic acid (22:6ω-3).

For fresh water fin fish the lipid profile of these nematodes without capelin enrichment appears promising, Kanazawa et al., (1980) mentioned that the growth promoting effects of 18:2ω-6 and 20:4ω-6 were superior to 18:3ω-3 and 20:5ω-3, indicating that ω6 fatty acids were more important nutritionally than ω3 fatty acids. Table 15 shows that nematodes grown on corn oil have very elevated levels of 18:2ω-6. Also levels of 20:4ω-6 are higher than that found in *Artemia*. Yone et al., (1978) had stated that ω3 HUFAs with carbon lengths greater than 20 played an essential role in the nutrition of marine fishes and are required in red sea bream.

Table 15. Comparison of fatty acid content in *P. redivivus* grown on media containing fish oil or corn oil to *Artemia*

Fatty acid	Artemia*	*P. redivivus* grown on 100% capelin oil	*P. redivivus* grown on 100% capelin oil	*P. redivivus* grown on 100% corn oil	*P. redivivus* grown on 100% corn oil
Lipid %	18.6+/-1.0	34.5	28	24	20.8
Protein %	59.5+/-2.2	55.9	53.4	51.3	50.4
14:0	6.261	4.86	4.87	0.1	0.09
16:0	9.902	11.1	11.07	8.95	9.15
16:1w7	12.964	5.42	5.18	0.52	0.55
17:0	0.468				
18:0	2.529	3.13	3.22	3.5	3.46
18:1w9	18.145	12.74	12.62	25.27	26
18:2w6	3.969	5.05	4.74	46.01	46.54
18:3n-3	3.031	0.87	0.87	0.86	0.98
19:0	0.721				
20:0	1.978	0.18	0.2	0.55	0.51
20:2w6	0.358	0.43	0.42	0.68	0.71
20:4w6	1.504	1.79	1.69	2.25	2.32
20:5w3	7.219	5.57	5.44	0.63	0.7
21:0	5.190				
22:6w 3	0.078	4.79	4.48	0.35	0.28

Artemia * denotes results taken from Biedenbach (1989).

CHAPTER THREE

PIGMENTATION OF THE NEMATODE *PANAGRELLUS REDIVIVUS*

Parts of this chapter have been published as follows:

Fisher, C.M. and Rodgers, P.B. 1993. Lipid enhancement and pigmentation of the nematode *Panagrellus redivivus* for use as an aquaculture feed. *Proc. of the World Aquaculture Society, New Orleans, Louisiana, U.S.A. 14-18 Jan, 1993.* Abstracts, pp.237.

3. PIGMENTATION OF THE NEMATODE PANAGRELLUS REDIVIVUS

INTRODUCTION

It is the characteristic colour of species such as salmon, shrimp and lobster that the consumer uses for identification of quality. This association of quality and colour is probably the most important aspect for marketing of aquaculture products and the producers of these products have a formidable market competitor, nature (Bird and Savage, 1990). The pink/red colour of *Salmo sp.*, and *Oncorhynchus sp* is of a general economic importance and whether these fish are farmed or caught wild they need to be pigmented to conform to consumer requirements. Pigmentation of salmon flesh is due to the depositions of oxygenated carotenoids. Similarly the market value of some penaeid shrimp depends directly on the intensity of the body redness.

Astaxanthin is the main pigment associated with the colour in prawns and is the pigment most easily utilised by shrimp. It is found in the carapace and epidermis of crustacea (Kuo et al, 1976; Omara-Alwala, 1985; Protasswicka, 1985). The major pigments of crustacean epidermal tissue are found in the monoester form while complex carotenoproteins found in the carapace result in a variety of blues and greens characteristic of live crustacea (Gomez et al, 1986). The red colour of cooked crustacea is produced by the release of the individual carotenoid prosthetic groups (astaxanthin) from carotenoproteins denatured by heat. Pigment supplementation into commercial crustacea diets is becoming increasingly common. Crustacea have the ability to synthesise the oxy and hydroxy derivatives of β carotene from the diet which have a definite advantage in absorption efficiency when compared to β - carotene (Yamada et al, 1990). Ingested carotenoids undergo oxidative metabolism and then are deposited in the epidermal tissues of the crustacea. Commercial pigmentation revolves around the shedding of the carapace material and must involve dietary supplementation of animals for a minimum of 2 complete moult cycles (10 weeks) to ensure adequate epidermal and carapace pigmentation.

Carotenoids represent a widespread group of natural pigments. They are synthesised de novo by all plants and some micro-organisms but they are also found throughout the animal kingdom, where they accumulate unchanged from the diet or are metabolically modified. Over 500

carotenoids are known to occur in nature. The structure is based on the hydrocarbons αcarotene, β carotene, and ϵ carotene. Carotenoids that contain oxygen functions are termed xanthophylls and the main components considered are zeanthin, lutein and astaxanthin.

Active redistribution of astaxanthin from the muscle tissue to the skin (male) and the ovary (female) in salmon led to the speculation that carotenoids have a role in salmon reproduction. Scheidt et al (1985) showed the conversion of astaxanthin to vitamin A in rainbow trout for diets deficient in Vitamin A. Steven (1948) showed that carotenoids were non essential by removing 90% of the oil droplets from the yolk sacs. Larvae developed normally, differing only in their pale colour and lack of characteristic colour pattern. From this it was concluded that the carotenoids do not have a physiological role in the development of juvenile trout and are present in eggs only to ensure correct development of species chromatophore pattern. Deufel (1965) fed female rainbow trout diets supplemented with canthaxanthin and reported enhancement of growth rate of maturation and fecundity of the fish. Some of the chemical characteristics of carotenoids may play an important role in the protection of the developing embryo.

Carotenoids may have a respiratory function when dissolved oxygen concentrations of the surrounding water are low (Baton, 1979 cited in Hard et al., 1989). White flesh strains of chinook salmon are present in British Columbia and South East Alaska characterised by a low muscle and ovarian pigment level. They spawn in cooler water ways and areas of high rainfall than their red fleshed cousins (Hard et al, 1989). This may indicate the white fleshed fish eggs are not adapted to survival in warmer waters with lower oxygen concentrations where their red fleshed counterparts successfully spawn.

Carotenoids also quench reactive oxygen species found following light irradiation (Burton, 1989). The most probable role of the carotenoids in fish and shrimp is the protection of the developing egg from photodynamic damage. High contents of carotenoids are detected in salmonid eggs, the adults of which appear to bury the eggs as they are sensitive to light (Mikulin, 1975). Torrissen (1984) recorded the mortality of eggs of Atlantic salmon irradiated with light of different wave lengths and concluded that egg development and the decrease in carotenoid concentration is related to the shift in light sensitivity towards the λ max of astaxanthin and

canthaxanthin. It therefore can be concluded that carotenoids play an important role in protecting lipid tissues from preoxidation in vivo as it provides protection against cancers by increasing the immune response. Since carotenoids are effective at low O_2 concentrations they may complement vitamin E which is effective at higher O_2 concentrations. Cold water fishes like salmon have high levels of polyunsaturated fats in their membranes, and protection of lipid tissues from peroxidation seems to be a possible function of astaxanthin or canthaxanthin in salmonids.

Carotenoids as mentioned above quench singlet oxygen and inhibit free radical initiation thereby decreasing the reactive product from natural oxido-reduction processes which can damage DNA and cell membranes (Burton, 1989; Bendich, 1989). Recently carotenoids were recognised as important immunological substances in mammalian studies. They execute their effects by interfering with the the various phases of carcinogenesis (Krinsky, 1989).

Sources

Astaxanthin is a major carotenoid found in many crustaceans. It is present in free and esterified forms and as protein complexes. Great effort has been made to evaluate different crustacean products as carotenoid sources for farmed fish. Crustacean wastes contain low levels of protein and high levels of moisture, ash and chitin which limits the percentage of these products that can be included in salmonid feeds. In Norway waste from shelling shrimp (*Pandalus borealis*) is the traditional natural pigment source for Atlantic salmon and rainbow trout. Krill (*E. superba, E pacifera*), the copepod (*C. finmarchicus*) and the red crab (*Pleuronectes planipes*)have been shown to pigment the flesh of salmonids. Norwegian commercial fish oil produced from capelin (*M. villosus*) contains considerable amounts of astaxanthin due to the intestinal contents of capelin. The oil can be extracted and incorporated into the feeds of rainbow trout.

Phaffia rhodozyma contains astaxanthin as it's principal carotenoid at 50 to 800 mg/kg depending on the strains and growing conditions. High astaxanthin levels makes *P. rhodozyma* a possible pigment source for salmonids. However lobsters (*Homarus americanus*) did not accumulate astaxanthin from *P. rhodozyma,* suggesting that yeast astaxanthin configuration cannot be as

integrated component of the carotenoprotein crustacyanin and therefore is of no value to lobsters (Shroeder and Johnson, 1993).

Paprika is a bright red colour mainly due to the capsanthin and capsorubin and has been known to pigment the flesh of salmonids. Lee et al., (1978) used extracts from the marigold flower (*Tagetes erecta*) and squash flower (*Cucurbita maxima marcia*). Total levels of carotenoids in the fish increases and the largest increase was seen in fish fed marigold flower extract. Marigold flower extract contains low levels of canthaxanthin.

Ketonic xanthophylls, echinenone, β,β-caroten-4-one; canthaxanthin and astaxanthin occur under favourable conditions as major components in green algae. Under unfavourable conditions i.e. nitrogen deficiency these carotenoids are synthesised in large amounts as secondary carotenoids. The ability to synthesise secondary carotenoids is almost exclusively restricted to the subphylum chlorophycae. The best known astaxanthin producing algae is *Chlamydomonas nivalis* which causes the red coloured snow at high altitudes on mountains in the summer. The algae is high in astaxanthin and produces pigmentation in atlantic salmon which is incorporated into the feeds at a level of 78mg/kg.

Synthetic Pigment Sources

Hoffman La Roche started commercial production of synthetic canthaxanthin in 1964 for colouring food and feeds. These are the dominant pigment sources used to colour cultured salmon, trout and shrimp species. The pigments are available in a stable dry beadlet form containing 10% astaxanthin or canthaxanthin. These are incorporated into commercial feeds.

AIMS

Experimental feeding trials with penaeid shrimp at Bangor University had shown that *P. redivivus* could be a potential replacement for *Artemia* in a number of penaeid diets (see chapter 5). However shrimp fed upon the nematodes looked paler compared to the control shrimp that fed upon the *Artemia*. It was therefore necessary to try and to incorporate the naturally occurring

pigment into the nematodes to produce a pigmentation of the shrimp similar to that of shrimp feeding upon *Artemia*.

The objective of the following experiments was to incorporate astaxanthin into the nutritional profile of the nematodes. With the final aim to feed pigment enriched nematodes to penaeid larvae to enhance pigmentation. All nematodes in the following experiments were cultured and harvested as described in chapter 1 using a medium containing 10% kidney, 3.5% corn oil and 1% yeast extract. Initially an analytical standard of astaxanthin was obtained from Roche products U.K. for calibration. In experiment 2 a sample of carophyll pink (8% astaxanthin) was obtained from Hoffman La Roche. It was found that the pigment dissolved in corn oil and water at 50^0C. However pigment was added directly to medium prior to autoclaving and dissolved easily whilst autoclaving. When nematodes were harvested the nematodes appeared to be pigmented. It was therefore necessary to investigate the amount of pigment that was taken up by the nematodes.

The experiment was designed to observe the growth of *P. redivivus* at 4 different pigment concentrations. The aim of the experiment was to see if increased concentrations of the pigment caused the increase in the pigmentation of the nematodes and affected the population numbers.

MATERIALS AND METHODS

P. redivivus nematodes were cultured as described in chapter 1 in the 'Materials and Methods section. All experiments were 1 x 4 factorial. All absorption readings were made at a wavelength of 478nm in a Philips TU8720 spectrophotometer. This wavelength as it showed peak absorption at all concentrations of astaxanthin dissolved in acetone. All samples of astaxanthin for spectrophotometric reading were calibrated against an acetone blank.

Experiment 1

Calibration curve for astaxanthin

An analytical standard of astaxanthin was obtained from Roche products U.K. The 50mg sample was dissolved in a series of dilutions of acetone and a calibrated absorption curve was established.

Experiment 2

Growth of *P. redivivus* in different concentrations of carophyll pink

A sample of carophyll pink (8%) astaxanthin was obtained from Hoffman la Roche. It was found that the pigment dissolved in corn oil and water at 50^{0}C. However pigment was added directly to medium prior to autoclaving as it dissolved easily whilst autoclaving. When nematodes were harvested the nematodes appeared to be pigmented under microscopic examination. It was therefore necessary to investigate the amount of pigment that was taken up by the nematodes.

The experiment was designed to observe the growth of *P. redivivus* at 4 different pigment concentrations. The aim of the experiment was to see if increased concentrations of the pigment caused the increase in the pigmentation of the nematodes and affected the population numbers. The following experimental conditions were used;

Bacteria; *Escherichia coli*

Temperature; 22^{0}C

Medium; 10% kidney, 3.5% corn oil, 1% yeast extract.

Flasks; 50ml containing 15ml of medium.

Concentration of pigment dissolved in water at 50^{0}C before adding to the medium: 0.1%, 0.005%, 0.002% and 0.001%.

Concentration of pigment in 15ml of medium; 0.6g/L, 0.3g/L 0.15g/L and 0.075g/L.

Pigment from the nematodes was extracted using a known volume of acetone and placed in a tissue homogeniser. The homogenate was then centrifuged and the supernatant decanted, made up to 10ml with acetone and standardised before measuring spectrophotometrically.

Experiment 3

Post harvest observation of pigment loss from *P. redivius* stored in water at 22^{0}C

This experiment was designed to observe the loss of this pigment over a period of time. The following culture conditions were use;

Bacteria; *Escherichia coli*
Temperature; 22^{0}C
Medium;10% kidney, 3.5% corn oil and 1% yeast extract.
Flasks; 50ml baffled containing 15ml of medium.
Inoculation; 2000 nematodes per ml.
Concentration of the pigment dissolved in the medium:5g/l, 10g/l, 15g/l 20g/l..

Nematodes were harvested at peak population levels (approx. 150 000 per ml). Nematodes were washed and aliquots of 1 million nematodes were placed into 15ml of clean water in 50ml baffled flasks sealed with cotton wool bungs and stored in shaking incubator at 22^{0}C.

On the first day that nematodes were harvested 4 samples of 1million nematodes were taken and homogenised with 5ml of acetone. The homogenate was then centrifuged and the supernatant collected and measured in a spectrophotometer for it's content of astaxanthin. Four samples were taken on days 2, 4, and 6.

Data analysis

Nematode population counts were compared by means of a statistical program SpSS for windows. Data was analysed by a one way ANOVA using Least Signicant Difference test with 95% confidence limits.

RESULTS

Experiment 1

The results presented in Fig. 1 assumed that a linear model was correct as this produced the linear regression equation **y = x108.43 - 0.0389**. Future unknown concentrations of astaxanthin can be extracted from the nematodes and calculated using this equation. Using these results approximate amounts of astaxanthin could be measured extracted from nematodes grown in media containing varying concentrations of Carophyll Pink.

Figure 1. Calibration curve for astaxanthin.

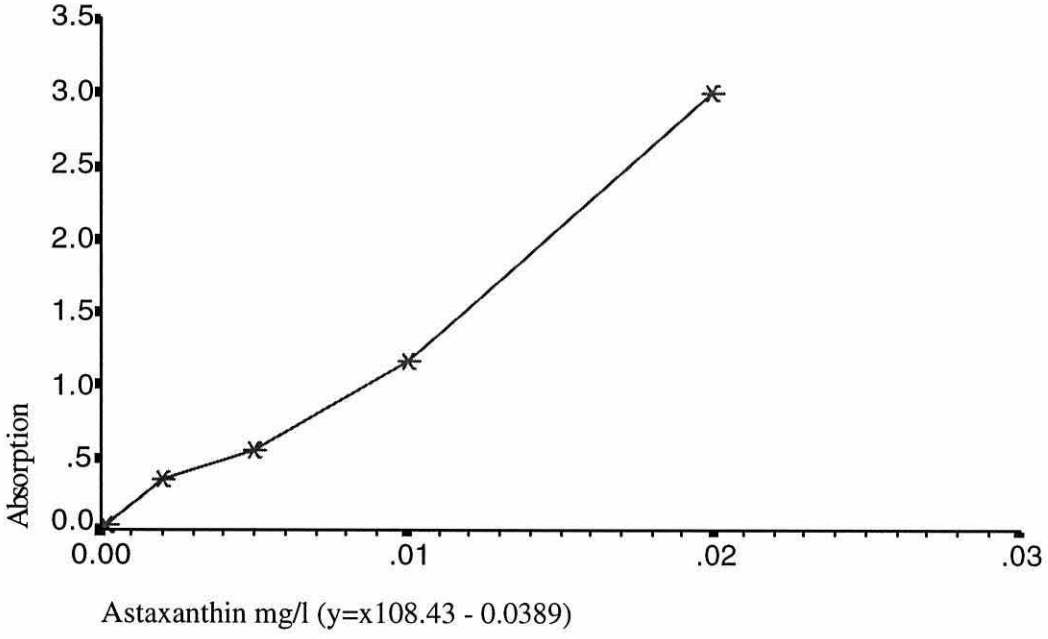


Table 1. Mean absorption of astaxanthin read from varying concentrations dissolved in acetone (3 replicates for each treatment).

Conc $mg^{-1}l$	0.02	0.01	0.005	0.002	0.0005
Absorption	3.00	1.16	0.56	0.36	0.04

Experiment 2

Growth of *P. redivivus* in different concentrations of carophyll pink

The results shown Table 2 shows that there is no significant difference between the growth rates and peak populations of nematodes grown on varying levels of pigment at the 5% level. After nematodes were extracted from the medium, the nematodes grown in 20g/l were very visibly pigmented. Nematodes grown on 10g/L and 15g/L were less pigmented and nematodes grown on 5g/L did not appear pigmented. Figs. 2 and 3 shows the posterior end of the nematode *P. redivivus* grown in a medium containing 20g/l and a medium not containing pigment. Figs 4 and 5 shows the anterior end of the nematode *P. redivivus* grown on a medium containing 20g/l of pigment and a medium not containing pigment.

Nematodes were harvested and analysed for their pigment content (Table 2). The results show that by increasing the concentration of the pigment in the medium the amount of astaxanthin present in the nematodes also increased. It was also noted that when the nematodes had been harvested and formulated in sponge the nematodes lost their pigmentation over a period of days. When nematodes are examined microscopically after harvesting from a pigmented medium the gut was seen clearly to contain the pigment. It was concluded that pigment is being retained in the gut of the nematodes and is actively being extruded when stored in foam blocks. It was not clear at this stage whether any of the pigment is incorporated into the tissues of the nematodes. Also Table 3 shows that a pigment concentration of 1.5% in the medium produces nematodes with a higher pigment content. These results are highly significant at the 5% level.

It was hypothesised that by increasing the carophyll pink concentration the level of astaxanthin in the nematodes would also increase. From the results it can be concluded that a concentration pigment of 2%(20g/l) does not increase the level of astaxanthin in the nematodes above that found in the lower concentrations. This could be due to the fact that a 2% concentration of carophyll pink saturates the kidney medium i.e. some of the pigment does not actually dissolve and and can be seen in the medium after autoclaving.

It therefore can be concluded that a maximum concentration of 1.5 % carophyll pink in the medium would be adequate to pigment the nematodes. By increasing this concentration the pigment remains undissolved and therefore wasted.

Table 2. *P. redivivus* grown on varying concentrations of carophyll pink

Concentration of carophyll pink (mg) in 1 l of medium					
Day	5g	10g	15g	20g	Significant
0	2000.00	2000.00	2000.00	2000.00	Ns
4	47375.00	61375.00	67625.00	70500.00	Ns
10	115250.00	106750.00	147000.00	119500.00	Ns
16	147500.00	140500.00	129000.00	128000.00	Ns
19	121750.00	117750.00	138000.00	117500.00	Ns

Table 3. Mean absorbency (4 replicates) of nematodes grown in different concentrations of carophyll pink.

Concentration of pigment dissolved in 1 L of medium	5g	10g	15g	20g
Absorbency of 1.8 million nematodes in 10ml of acetone	0.98 0.93 0.94	1.26 1.32 1.21	1.49 1.39 1.58	1.29 1.42 1.30
Mean absorption	0.95	1.26	1.49	1.34
Astaxanthin content in 1.8 million nematodes in 10ml acetone (mg)	0.01	0.012	0.014	0.013
*Astaxanthin content (mg) in 1million wet weight nematodes	0.005	0.006	0.007	0.007

* Calculations worked out from the line of best fit for the absorption of acetone in astaxanthin,= **y = x108.43 - 0.0389** (Fig. 1).

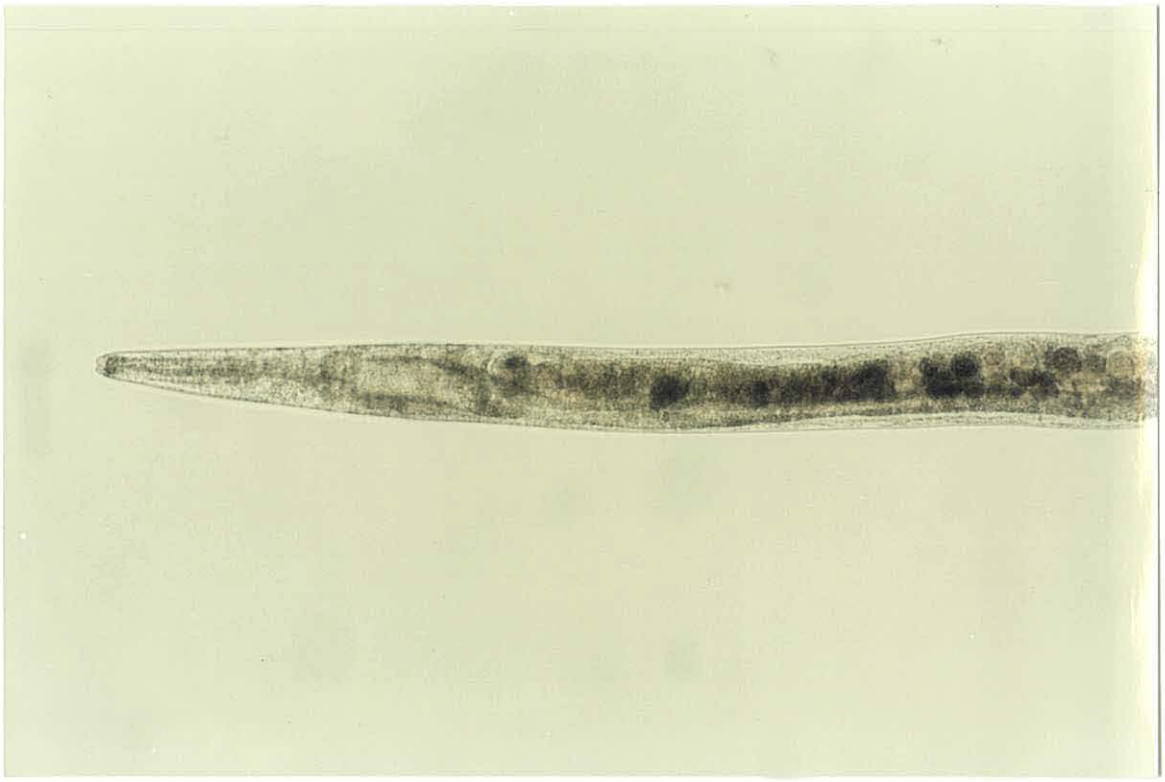


Figure 2. Posterior end of the nematode *P. redivivus* grown in a medium not containing pigment x 200.

Figure 3. Posterior end of the nematode *P. redivivus* grown in a medium containing 20g/l of pigment x 200.

Figure 4. Anterior end of the nematode *P. redivivus* grown in a medium not containing pigment x 200.

Figure 5. Anterior end of the nematode *P. redivivus* grown on a medium containing pigment 20g/l x 200.

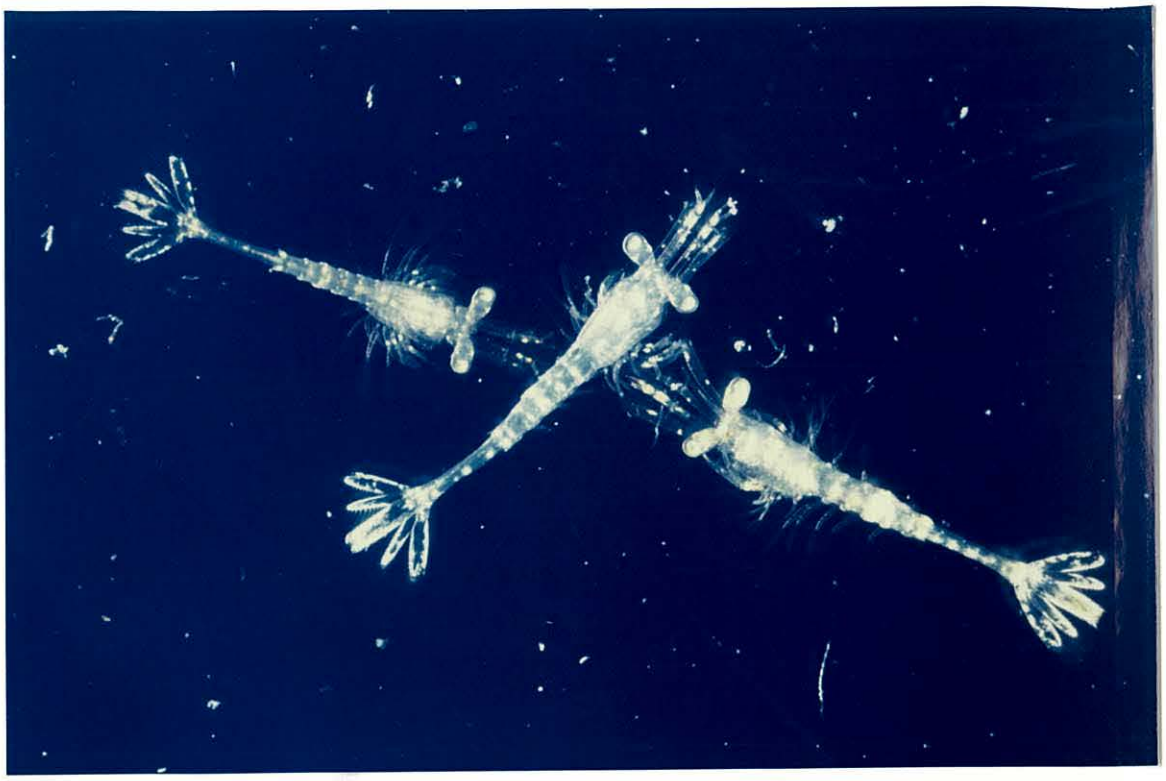


Figure 6. *Penaeus indicus* larvae at the PL stage (x 15) showing no pigmentation after being fed non pigmented nematodes from experiment 1, chapter 6.

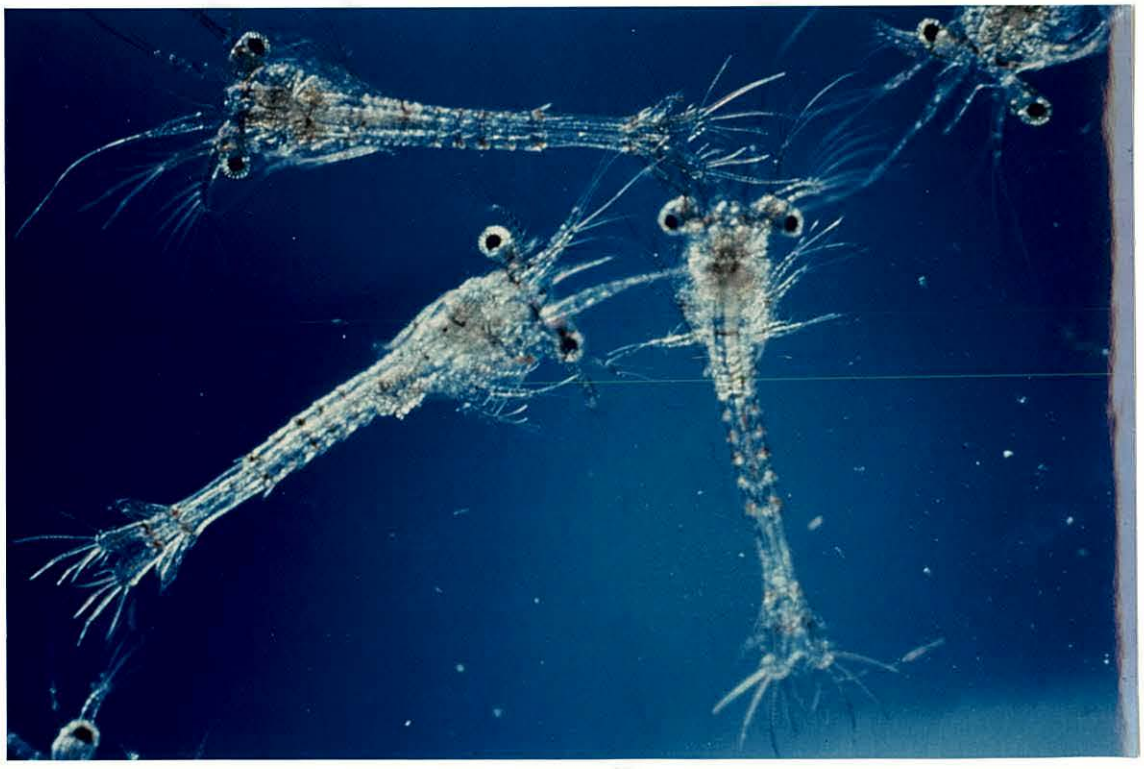


Figure 6. *Penaeus indicus* larvae at the ZIII stage (x 40) showing clear pigmentation after being fed with pigmented nematodes from experiment 1, chapter 6.

Experiment 3

Post harvest observation of pigment loss from *P. redivius* stored in water at 22^{0}C

From the results Table 4 it can be concluded that pigment is lost from the nematodes if they are stored in water at 22^{0}C for up to 6 days. Pigment was not detected in nematodes on day 6 or there after.

Table 4. Astaxanthin content in 1g of dry weight nematodes is calculated from the best line of fit equation calculated for the absorption of astaxanthin in acetone.

Day	0	1	2	4	6
*Absorption	1.49	1.15	0.48	0.41	0
	1.39	0.72	0.57	0.49	
	1.58	0.84	0.31	0.53	
	-	0.96	0.39	0.33	
Mean absorption	1.49	0.92	0.44	0.44	0
Astaxanthin present (mg) in 1million nematodes wet weight	0.007	0.004	0.002	0.002	0

* Nematodes harvested and used from Experiment 2 (Table 2).

DISCUSSION

The addition of pigment to the growth medium has no effect on the development of the nematodes. Nematodes grown in a medium containing 0.6g/L, 0.3g/L and 0.15g/L readily took up the pigment. At weaker concentrations, nematodes were unable to pick up the pigment. It was clear from microscopic observation and photographic evidence (figs. 2,3,4 and 5) that pigment is deposited in the guts of the nematodes and if stored at 22^{0}C on sponge blocks it is actively extruded from the guts. Table 2 indicates that pigment does not remain on the gut beyond 4 days

at 22^0C.

It was also possible to increase the level of pigmentation in the nematodes by increasing the concentration of pigment added to the growth medium. Table 3 shows that a maximum concentration of pigment that can be added to the growth medium was 1.5%. Above this concentration nematodes were unable to increase the pigment concentration in their guts. It is not clear whether or not pigment is incorporated into the tissue of the nematodes. Figures 6 and 7 show the appearance of *P. indicus* larvae fed pigmented and non pigmented from experiment 1, chapter 6. It is clear from these results that pigment can be incorporated by the larvae after being fed pigmented nematodes.

CHAPTER FOUR
FORMULATION AND STORAGE

4. FORMULATION AND STORAGE

INTRODUCTION

It is important that nematodes can be stored and transported for extensive periods of time if they are to have a possible use on a commercial scale. At the present time only entomopathogenic nematodes are used commercially and formulated using a variety of techniques (Table 1.). The majority of formulation and application studies have been undertaken with insect parasitic nematodes, which are known to have in their life cycle an infective stage or resting juvenile (dauer). The nematodes in this stage can survive in the soil for extended periods of time under conditions such as desiccation and exposure to ultraviolet radiation and high temperatures.

Entomopathogenic nematodes can be formulated in a variety of the following substances: a) alginate, b) polyether-polyurethane sponge, c) gel-forming polyacrylamides, d) clay, e) vermiculite and peat, f) evaporetardants and ultraviolet protectants, g) anhydrobiotic nematodes, h) baits.

Nematodes when formulated require a large surface to volume ratio for oxygen exchange, when storing nematodes, the following factors must be considered:

1) Requirement of refrigeration.
2) Requirement of oxygen (provided through aeration and airspace).
3) Limited nematode density.
4) Difficulty of extraction of nematodes from the packaging.

There is limited literature available on the formulation and storage of free living species of nematodes. The majority of the techniques that have been applied to entomopathogenic nematodes could be applied to free living species. Little is known about the optimum storage conditions for the nematode *P. redivivus*. The nematode does not form a resistant infective stage as it is free living and hence it is necessary to immobilise the nematode in a matrix to prevent depletion of the energy stores. Therefore it is necessary to investigate suitable storage components

Table 1. Summary of nematode formulation for the use in the agricultural industry.

FORMULATION TECHNIQUES		
Formulation	Nematode	Authors
Alginate beads	*S. feltiae*	Kaya and Nelsen,(1985); Redenbaugh et al.,(1984); Fravel et al, (1985); Lackey et al, (1993); Poinar et al, (1985); Nelsen et al, (1985); Paparizas, Fravel and Lewis, (1987); Bashan, (1986); Walker and Connick, (1983); Kierstan and Bucke, (1977); Lewis and Papavizas, (1985).
Polyether polyurethane sponge	*Steinernema spp.* *S. bibionis* *S. glaseri*	Bedding (1984)
Gel forming polyacrylamides	*S. carpocapsae*	Schroid (1990)
Clay, vermiculite and Peat	*S. feltiae* *H. megidis* *P. hermaphrodita*	Godliman (1983, 1991 and 1994) unpublished data. Connick Jr (1991)
Anhydrobiosis	*Ditylenchus spp.* *S. feltiae* *Heterorhabditis spp.* *S. carpocapsae*	Barret (1987) Womersley (1987)
Bait formulation	*S. carpocapsae*	Georgis, (1990)

AIMS.

Nematode storage survival was initially investigated by undertaking short term high temperature storage experiments to assess optimum water content, sponge weights and nematode numbers. The aim of experiment 1 was to assess the optimum amounts of water and sponge needed for the storage of *P.redivivus*. Experiment 2 assessed the survival of 5 and 10 million nematodes which were investigated in varying amounts of foam chips. Experiment 3 was undertaken to investigate optimum amounts of water needed for treatments of nematodes stored in foam chips.

From the above experiments it was established that 5 million nematodes could be stored with either 2g or 3g of foam chips and 10ml of water. 5 million nematodes were used in experiment 4 due to limiting amounts of nematodes available for the quantites required for this experiment. Nematodes were stored at 5^0C and the lipid and protein depletion was observed during storage to determine the nutritional quality of the nematodes over time.

It was established lipids and proteins were depleted when stored in foam chips at 5^0C. It was therefore necessary to find an alternative inert carrier that would reduce nematode losses and also prevent the decline in lipid and protein content. Alginate beads were thought to be a promising carrier matrix for *P. redivivus* and were investigated. The objective of experiment 5 was to observe the optimum number of nematodes that could be stored in alginate beads. Four concentrations of nematodes were investigated; 200, 300, 400 and 600. Up to 1000 *S. feltiae* can be embedded in alginate beads by Poinar et al (1985). Lower figures were chosen for *P. redivivus* as these nematodes were larger and very active.

From the above experiment it was established that optimum numbers could be stored in alginate capsules. Experiment 6 explores the overall survival of nematodes stored in alginate capsules at 22^0C containing optimum numbers and to observe how survival at the same temperature compared with nematodes stored previously in foam chips in bags.

The objective of experiment 7 is to incorporate increased numbers of nematodes into the beads. It was established from experiment 6 that a maximum number of nematodes could be incorporated into the capsules was 200 stored at 22^0C for 35 days. It was concluded that the vigorous movements of the nematodes were causing damage to themselves as the sodium alginate beads were solidifying in the gelling agent hence it was not possible to increase above 200 nematodes per bead. In this experiment all reagents were chilled to approximately 0^0C to restrict the movement of the nematodes prior introducing the nematode paste into the chilled Sodium citrate.

Experiment 8 explored the survival of nematodes at the 4 concentrations described in the previous experiment at 5^0C. Following on from this experiment establishing the optimum

number of nematodes in capsules an experiment was set up to investigate maximum temperatures that the capsules containing nematodes could be stored at. 3 temperatures were investigated; 22^0C, 18^0C and 5^0C.

It was thought that as stored capsules with nematodes lost water this might affect the accuracy of the nematode counts . An experiment was set up to compare the differences in nematode counts using two methods 1) counting a known number of beads and extracting the nematodes from this number 2) measuring a known amount of alginate-nematode complex and extracting the nematodes.

Following on from experiments undertaken in chapter 3. An experiment was set up to observe the pigment loss (astaxanthin) from nematodes stored at room temperature (22^0C) and formulated in alginate beads. It was assumed that the prevention of movement of the nematodes in the beads would prevent the loss of pigment Experiment 11 investigates this possibility. Finally the loss of pigment from nematodes stored in alginate capsules was investigated at 5^0C. However the removal of the spectrophotometer meant that nematodes were visually scored for the loss of pigment. The nematodes containing pigment were easily identified due to a dark red pigment in their guts.

MATERIALS AND METHODS

Experiment 1

Survival of nematodes in polyethylene foam chips at 22^0C.

20, 15, 10 and 5 million nematodes were packed into high density polyethylene bags (15cm x 15cm) containing 2g sponge and 10ml water and stored at 22^0C for a period of 12 days. The objective of this experiment was to determine the optimum number of nematodes to be stored in sponge chips. By undertaking the experiment at 22^0C the effects of storage on the nematodes is enhanced. All nematodes for experiments were cultured using the following method;
Bacteria; *Escherichia coli*

Temperature; 22^{0}C

Medium; 3.5% kidney, 5.95% egg, 3.5% oil (50% capelin oil and 50% corn oil), 0.78% yeast extract.

Vessel; 10L fermenter

Inoculation level; 2000 nematodes per ml.

Nematodes were harvested after 14 days.

Experiment 2

Survival of 5 million *P. redivivus* in 3g, 2g, 1g and 0.5g of sponge stored at 22^{0}C.

Nematodes were packed into high density polyethylene bags containing 3g, 2g, 1g and 0.5g with 10ml of water. The objective of these experiments was to determine the optimum weight of sponge needed to store these quantities of nematodes.

Experiment 3

Survival of 10 million *P.redivivus* in 2g of foam chips containing 15ml, 10ml and 5ml of water at 22^{0}C.

10 million nematodes were packed as described previously in experiment 2 with 3 different amounts of water 15, 10, and 5ml. Four replcates for each treatment were made. The treatments were stored at 22^{0}C.

Experiment 4

Survival of *P. redivivus* stored in 2g foam chips with 10ml of water at 5^{0}C.

Five million nematodes were packed in polyethylene bags containing 10 ml of water and 2g of foam chips. Bags were sealed and placed at 5^{0}C. Four replicates were analysed at intervals over a period of 10 weeks. Lipid analysis was also undertaken (see chapter 2 for method). The main objective of this experiment was to observe the lipid and protein depletion in the nematodes stored for a long period at a low temperature and determine the nutritional quality over this time.

Experiment 5

Survival of *P. redivivus* at concentrations of 200, 300, 400 and 600 nematodes per bead at 22^0C.

To prepare the sodium alginate beads containing different concentrations of nematodes the following method was used; a 2% solution of sodium alginate was mixed with a paste of nematodes (known weight and numbers). The preparation was added drop wise to a 1.47% solution of $CaCl_2$. The capsules were then left to complex with the solution for a period of 20 min. The nematodes were observed to congregate in the centre of the beads. The diameter of the beads averaged approximately 3mm. The number of nematodes in the beads could be altered by changing the amount of nematode paste added to the alginate solution.

Survival of the nematodes was assessed by removing 10 beads and placing them into a 10ml 0.1m solution of sodium citrate. With vigorous shaking the beads dissolved within 10 min. Nematode numbers per bead could then be assessed by taking 1ml samples and dissolving them into 500ml of water, this was continuously aerated. Four samples were taken for each treatment, counts were made in a 1ml graded counting chamber.

Experiment 6

Survival of *P. redivivus* in alginate beads stored at 22^0C.

After establishing the optimum numbers of nematodes that could be stored in alginate capsules an experiment was set up to observe the survival of nematodes stored at 200 nematodes per bead at 22^0C over a period of time. To incorporate nematodes into sodium alginate capsules the same method was applied as described above. Capsules were placed on a moist filter paper in covered petri dishes. Nematode numbers were assessed using the method described above.

Experiment 7

Survival of *P. redivivus* stored in alginate beads in concentrations of 10 000, 6000, 4000 and 2000 nematodes per bead at 22^{0}C.

Four known weights of nematode paste were chilled and mixed separately into chilled solutions of sodium alginate. The sodium alginate solution was made by mixing 2g of sodium alginate into 100ml of water. These solutions added drop wise into a chilled gelling agent. The beads were left on ice to form for 20 mins. The beads measuring approximately 3mm in diameter were placed in glass petri dishes at 22^{0}C. Nematode numbers were assessed at intervals over a period of 30 days.

Experiment 8

Survival of *P. redivivus* stored in alginate beads in concentrations 10 000, 6000, 4000 and 2000 nematodes per bead at 5^{0}C.

As with experiment 7.

Experiment 9

Survival of *P. redivivus* stored in alginate beads at 4 concentrations below 4000 nematodes per bead and stored at 5^{0}C, 18^{0}C and 22^{0}C.

Four known weights of nematode paste were chilled and mixed separately into chilled solutions of sodium alginate. The sodium alginate solution was made by mixing 2g of sodium alginate into 100ml of water. These solutions were added drop wise into a chilled gelling agent. The beads were left on ice to form for 20 min. The beads measuring approximately 3mm in diameter were placed in glass petri dishes at 5^{0}C, 18^{0}C and 22^{0}C. Nematode numbers were assessed at intervals over a period of 30 days. Treatment **A** contained an average of 1163 nematodes per bead, Treatment **B** contained an average of 1688 nematodes per bead, Treatment **C** contained an average of 2038 nematodes per bead and Treatment **D** contained an average of 3888 nematodes per bead. The experiment aimed at obtaining treatments containing approximately 1000, 2000, 3000 and 4000 nematodes per bead. However it was very difficult to obtain exact numbers in

the beads.

Experiment 10

Survival of *P. redivivus* stored in 50g bags of alginate beads.

Differences in nematode counts were compared using two methods: 1) counting a known number of beads and extracting the nematodes from this number, 2) measuring a known amount of alginate-nematode complex and extracting the nematodes. The nematode alginate complex was prepared earlier as described in previous experiments. Alginate beads were then stored in high density polyethylene bags at 5^0C containing 50g of alginate complex. Samples were taken at regular intervals, either by removing known numbers of beads or weights of alginate.

Experiment 11

Loss of pigment from alginate beads stored at 22^0C.

Nematodes were harvested from 50ml shake flasks containing the standard shake flasks medium enriched with 1.5% astaxanthin as described in chapter 3. A known number of nematodes were formulated into alginate beads. Approximately 1000 nematodes per bead were obtained per bead. The proportion of alginate to nematode weight was also measured, 30g of alginate = 1 million nematodes.

Alginate beads were stored at 22^0C in plastic trays. At selected intervals over a seven day period nematodes were extracted from the beads and measured for pigment loss. 1 million nematodes were extracted and macerated with 10 ml of acetone. Nematode fragments were removed by centrifugation and filtration. The supernatant was made up to 10ml (loss from evaporation) and measured spectrophotometrically at 478nm for the astaxaxanthin content.

Experiment 12

Loss of pigment from nematodes stored in alginate beads and foam chips.

Due to the removal of the spectrophotometer, the loss of the pigment from nematodes stored in alginate beads using the same concentration of nematodes as those found in experiment 11 was

assessed visually. The nematodes were easily identified due to a dark red pigment in their guts. Nematodes were cultured using the same conditions as described in the previous experiment. 100 nematodes were scored using bench microscope examination for a period of 12 days.

RESULTS

Experiment 1

Survival of nematodes in polyethylene foam chips at 22^{0}C

Fig.1 shows that treatments containing 10 million and 5 million nematodes survived the longest. Nematodes stored in bags containing 15 and 20 million were rapidly fouled after 4 days due to the high density of nematodes in the packs. Therefore for the following experiments treatments of 5 and 10 million nematodes were chosen.

Table 1. Mean nematode counts from 4 samples in polyethylene foam chips.

	Days					
0	2	4	6	8	10	12
$20x10^6$	19105250	17383750	1144175	9194000	2243250	0
$15x10^6$	14528000	13321500	8395000	2379500	38025	0
$10x10^6$	8742500	7652000	7107500	4771750	3329000	1060000
$5x10^6$	4046750	3452500	2437750	1497500	153250	40250

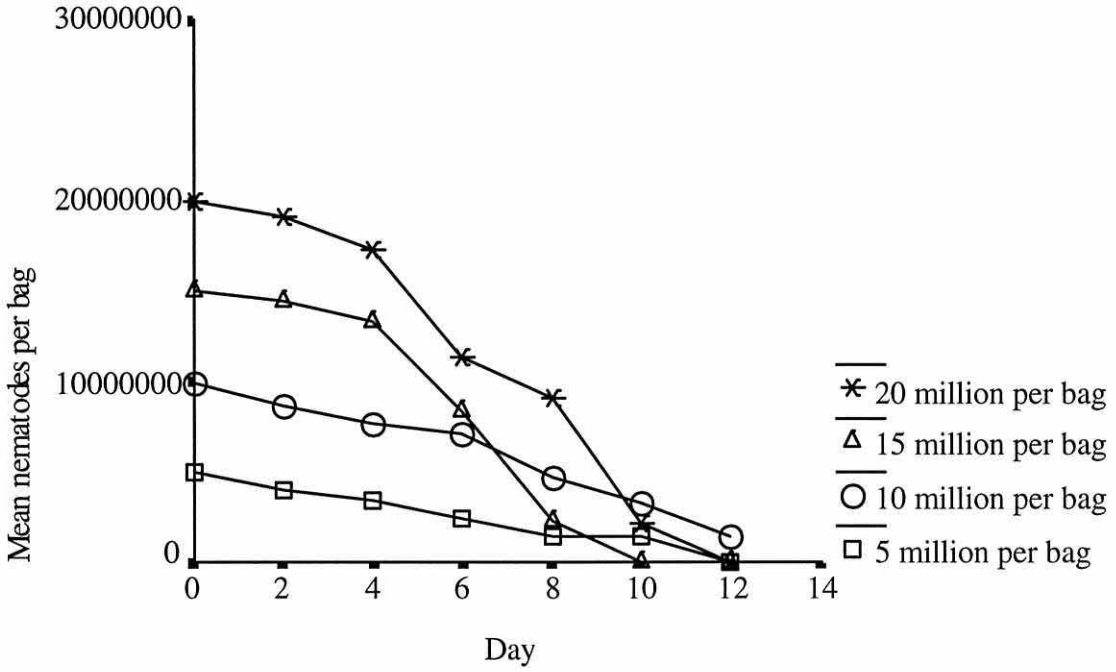


Figure 1. Survival of 20 million, 15 million, 10 million and 5 million nematodes in polyethylene foam chips at 22^{0}C.

Experiment 2

Survival of 5 million *P. redivivus* in 4 weights of foam chips stored at 22^{0}C.

The results in figure 2 show that on day 4 the 3g treatment was significantly different from the 0.5g treatment at the 5% level. On day 2 the 2g treatment is significantly different from 1g and 0.5g treatments at the 5% level. In this experiment 5 million nematodes stored in 2g and 3g had a superior survival.

Table 2. Survival of 5 million *P. redivivus* in 4 weights of foam chips stored at 22^{0}C (mean counts).

	Days			
Foam weights (g)	0	4	7	10
0.5	5000000	3140000	1660666	3433
1.00	5000000	3626666	673333	9000
2.00	5000000	3473333	1493333	80333
3.00	5000000	3886666	1586666	39333

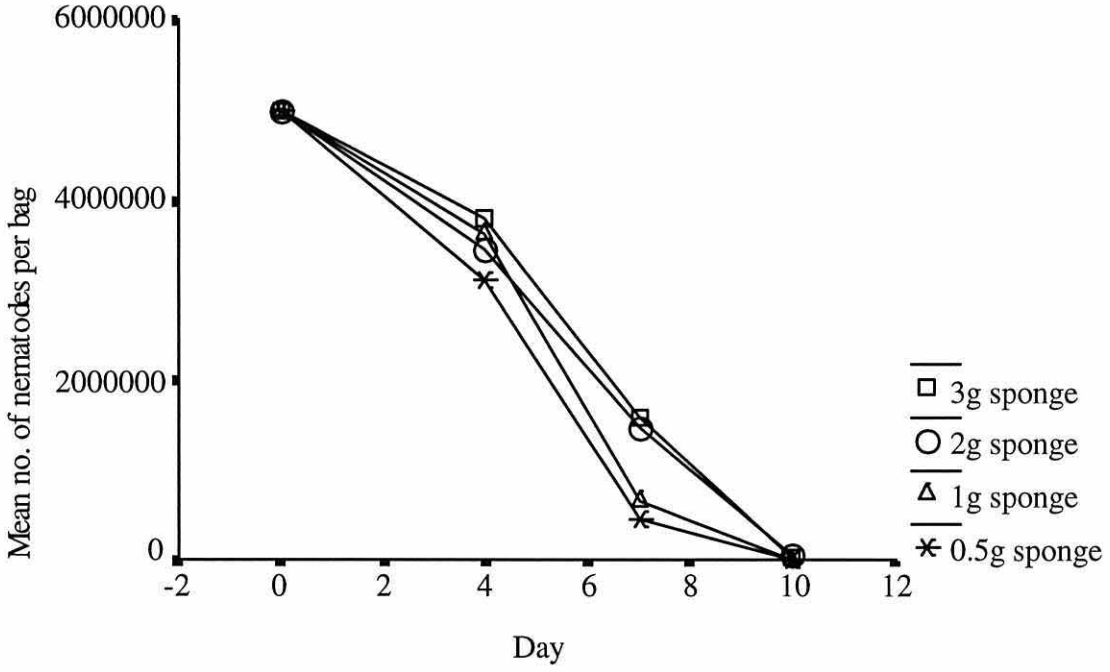


Figure 2. Survival of 5 million *P. redivivus* in 3g, 2g, 1g and 0.5g of sponge stored at 22^{0}C

Experiment 3.

Survival of 10 million *P . redivivus* in 2g of foam chips containing 15ml, 10ml, and 5ml of water at 22^{0}C.

Results in Table 3 show that no significant differences were observed at day 1 between treatments in figure 3. On day 2 and day 6 the treatment containing 3g of sponge was significantly different from all the other treatments. On day 8 treatments containing 3g and 0.5g of sponge were significantly different from all the other treatments at the 5 % level. The packs containing 3g of nematodes appeared dried out which would explain the apparent poor survival and quality of the nematodes. Treatments containing 0.5g of sponge were fouled due to the high concentration of nematodes to sponge.

Figure 3 shows that significant differences were observed for treatments at day 4, 6 and 10 at the 5 % level. Superior survival was observed with treatments containing 10ml and 5ml of water. After 10 days all treatments containing 15 ml of water were fouled.

Table 3. Survival of 10 million *P . redivivus* in foam chips at 22^{0}C (mean counts).

	Days					
Foam weights (g)	0	1	2	6	8	10
0.5	$1x10^6$	9466666	8933333	2533333	2493333	0
1.0	$1x10^6$	$10.16x10^6$	9533333	7866666	4653333	29666
2.0	$1x10^6$	9000000	8766666	7733333	3773333	21000
3.0	$1x10^6$	9500000	2000000	683333	20546	0

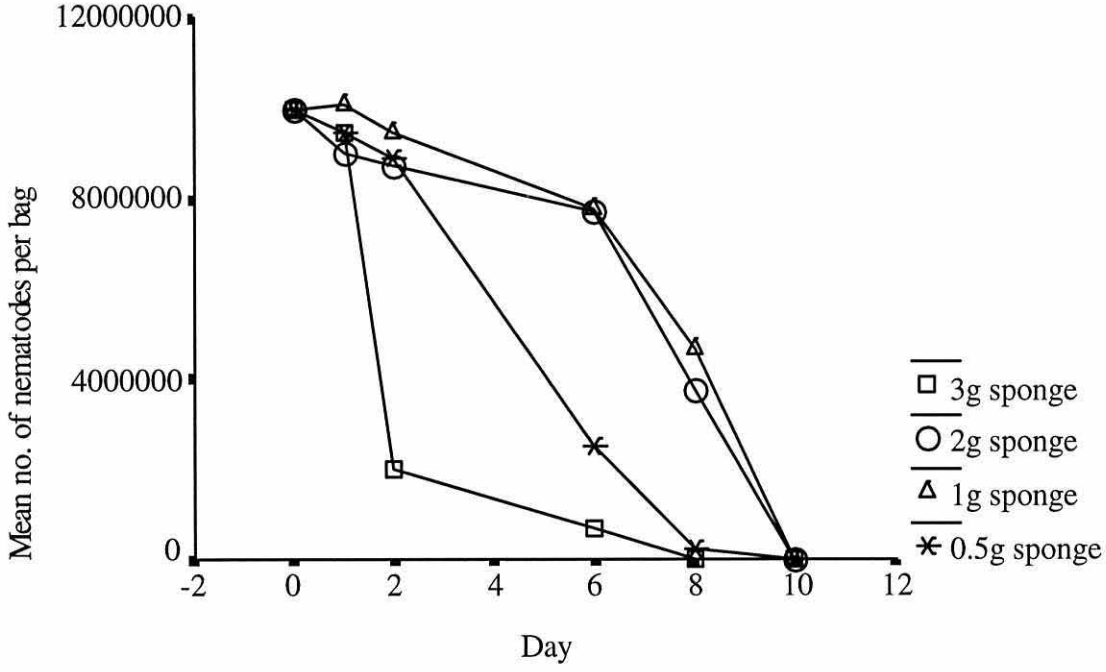


Figure 3. Survival of 10 million nematodes stored in 3g, 2g, 1g and 0.5g of polyethylene sponge at 22^0C.

Figure 4 shows that for treatments at day 4, 6 and 10 at the 5% level. Superior survival was observed with treatments containing 10ml and 5ml of water. After 10 days all treatment containing 15 ml of water were fouled.

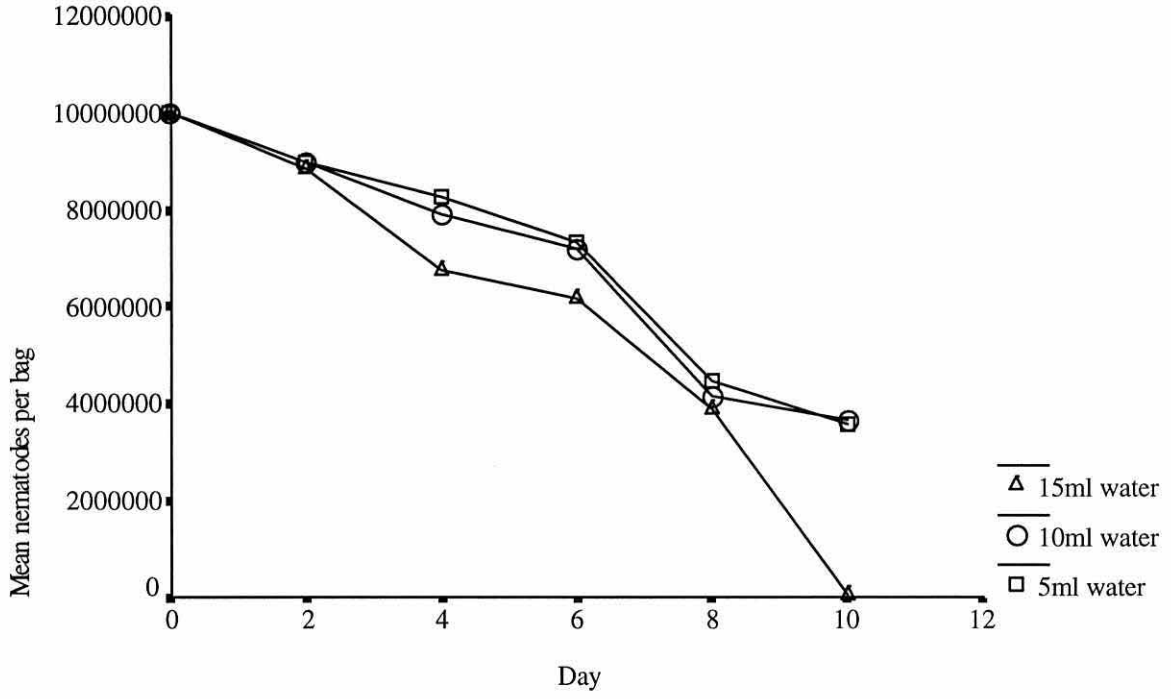


Figure 4. Survival of 10 million nematodes stored in 2g of foam chips with 15,10 and 5 ml of water at 22^{0}C

Experiment 4

Survival of P. redivivus stored in 2g foam chips with 10ml of water at 5^{0}C

The results in Figure 5 clearly show a gradual decline in the population levels of nematodes stored in the foam chips at 5^{0}C. There is a significant drop in numbers at the 5% level. Significant differences were observed in populations in bags stored from weeks 2-5. Significant differences were observed between treatments stored from weeks 5 up to 10 weeks to those from weeks 1 to 5. Treatments stored up to 10 weeks had a population significantly lower at the 5% level than all other treatments from previous weeks, these bags were also foul smelling.

The nematode's visual quality declines after a period of 3-4 weeks. The nematodes appear transparent and do not produce vigorous movement. After 10 weeks nematodes are of an extremely poor quality. The results indicate a maximum period of storage of 4 weeks in foam chips at 5^{0}C.

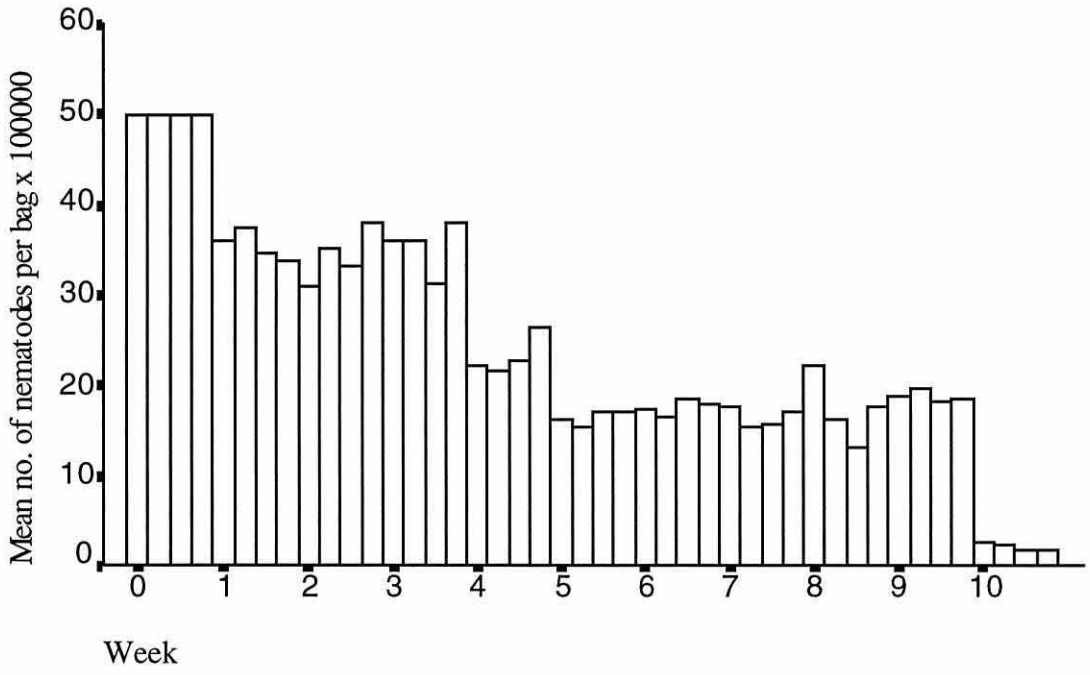


Figure 5. Survival of *P. redivivus* stored in 2g of foam chips with 10ml of water at 5^0C.

Table 4. Mean of 4 replicates, survival of *P. redivivus* stored in 2g of foam chips with 10ml of water at 5^0C for 10 weeks.

Week	0	1	2	3	4	5	6	7	8	9	10
Mean x 10^6	5	3.55	3.44	3.54	2.33	1.65	1.76	1.64	1.73	1.89	2.18

Nematodes were also analysed for their lipid and protein content. The results in Table 5 show a decline in total lipid content which then remains relatively stable until week 10 this is reflected by the decrease in numbers shown in fig. 5. Protein content remains constant. A decrease in fatty acids over period of 10 weeks was observed in the following fatty acids (14:0, 18:1ω-9, 18:4ω-3, 20:1ω-9, 22:6ω-3 and 22:1) an increase was observed in (18:0, 20:4ω-6 and 20:5ω-3) (Table 5). It was noted that the percentage saturated fatty acids increased and monounsaturated fatty acids decreased , with monosaturated decreases in 16:1ω-9, 18:1ω-9, 20:1ω-9 and 22:1ω-11, increase in the saturated fatty acids corresponded with 18:0. The total remains the same, suggesting a conversion between saturated and monosaturated fatty acids or a depletion in the lower chain fatty acids. A possible energy source for the nematode could therefore be 18:1ω-9,

18:2ω-6, 18:3ω-3 and 18:4ω-3.

Table 5. Total lipid, fatty acid levels in *P. redivivus* stored in foam chips for 10 weeks.

Week	0	1	2	4	5	6	7	8	9	10
Lipid %	18.9	15.3	15.4	12.5	11	12.1	9.3	11	9	3.5
Protein %	48.9	44.8	46.7	53.6	46.5	38.8	51.8	42.3	47.7	51.5
14:0	0.76	0.58	0.35	0.52	0.48	0.45	0.51	0.41		0.39
16:0	4.04	3.99	3.65	4.11	3.96	3.4	5.13	3.23	3.63	3.58
16:1ω-9	15	12.5	13.1	11.7	11.8	11.2	13.1	10.4	9.78	10.4
18:0	3.82	4.79	5.06	5.58	6.13	6.41	5.78	6.73	7.25	7.32
18:1ω-9	15	12.5	13.1	11.7	11.8	11.2	13.1	10.4	9.78	10.4
18:1ω-7	5.14	4.76	5.05	5.99	6.43	5.58	5.56	5.41	5.92	5.96
18:2ω-6	28.7	24.9	26.2	23.1	26.1	24.4	27.2	23.4	21.5	23
18:3ω-3	0.7	0.86	0.85	0.46	-	0.45	0.58	0.5	0.47	0.59
18:4ω-3	0.74	0.73	0.74	0.48	-	0.54	0.54	0.47	0.52	0.55
20:0	0.24	0.41	0.32	0.38	0.34	0.23	0.3	0.39	0.42	0.3
20:1ω-9	4.92	4.32	4.48	4.17	4.18	3.96	3.68	3.94	3.69	3.81
20:2ω-6	1.34	1.72	1.79	1.77	1.72	1.7	1.54	1.7	1.63	1.79
20:3ω-6	1.61	2.51	2.65	2.91	3.13	3.43	2.86	3.6	3.6	3.65
20:4ω-6	2.28	3.46	3.66	4.01	4.32	4.52	4.1	5.01	5.28	5.36
20:4ω-3	0.87	1.41	1.33	1.43	1.19	1.24	1.15	1.27	1.28	1.25
20:5ω-3	**8.01**	**9.72**	**10.7**	**10.9**	**11.5**	**11.7**	**11**	**12.5**	**12.1**	**12.2**
22:1ω-11	5.12	3.93	4.13	4.07	3.49	3.33	2.91	2.98	2.73	3.06
22:5ω-3	0.3	0.23	0.15	0.11	0.13	0.2	0.13	0.2	0.15	0.17
22:6ω-3	**2.36**	**1.68**	**1.72**	**1.41**	**1.49**	**1.38**	**1.44**	**1.13**	**1.01**	**1.01**
% Sats	8.86	9.77	9.38	10.6	10.9	10.5	11.7	10.8	11.3	11.6
% MonoUn	34	28.8	29.7	29	28.7	27.1	27.7	25.3	24.3	25.9
% PolyUn	46.9	47.2	49.8	46.5	49.6	49.5	50.6	49.8	47.6	49.6
TOTAL	89.8	85.7	88.9	86	89.2	87.1	90	85.9	83.2	87
Omega-3	15.3	14.6	15.5	14.8	14.3	15.5	14.8	16.1	15.6	15.8
Omega-6	33.9	32.5	34.3	31.7	35.3	34	35.7	33.7	32	33.8
rat ω-6/ω-3	2.22	2.22	2.21	2.15	2.48	2.19	2.41	2.09	2.06	2.14

Experiment 5

Survival of *P. redivivus* at concentrations of 200, 300, 400 and 600 nematodes per bead at 22^{0}C.

The results of this experiment (Figure 6) show that at room temperature nematodes survive better when stored with approximately 200 nematodes per bead. These results are significantly different at the 5% level from nematodes stored at 300, 400 and 600 nematodes per bead. It can be concluded that the optimum number of nematodes per bead when storing at 22^{0}C is 200. The larger size of the nematode and it's vigorous movement may prevent higher numbers being stored in the capsules as this may cause damage to the nematodes.

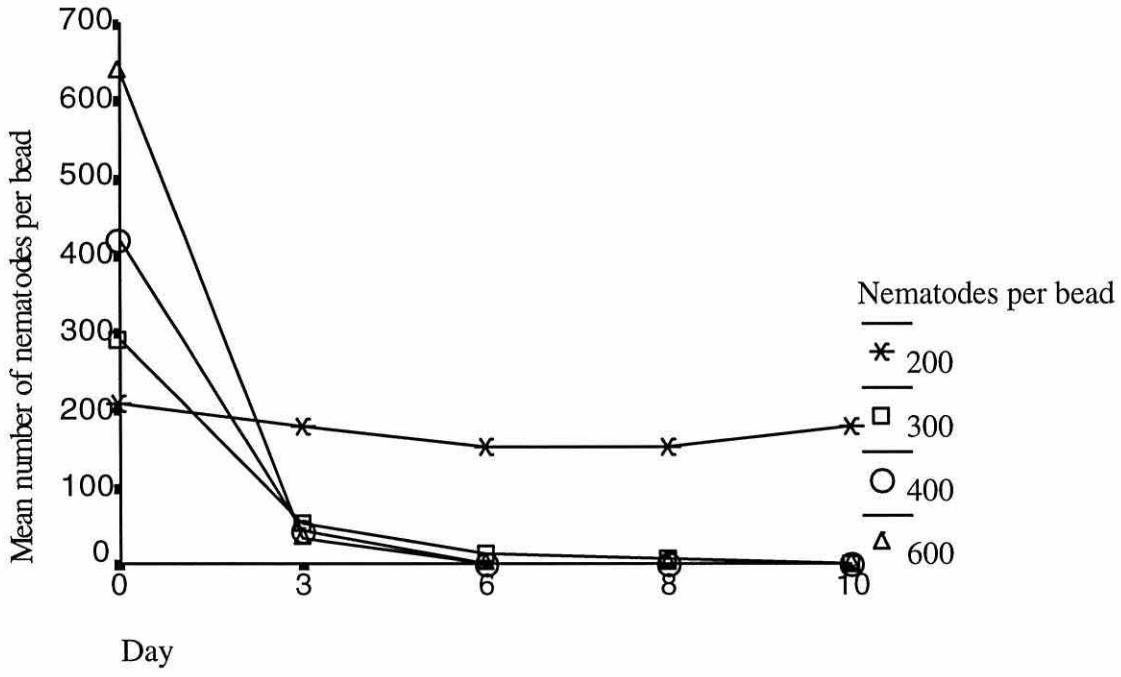


Figure 6. Survival of *P. redivivus* at concentrations of 200, 300, 400 and 600 nematodes per bead at 22^{0}C.

Table 6. Mean of 4 replicates Survival of *P. redivivus* at concentrations of 200, 300, 400 and 600 nematodes per bead at 22^{0}C.

Treatments					
Day	200	300	400	600	Significant
0	211.00	293.50	293.50	639.25	*
3	181.50	55.5	43.50	33.50	*
6	152	13.50	0.00	0.00	*
8	152	8.00	0.00	0.00	*
10	181	0.00	0.00	0.00	*

Experiment 6

Survival of *P. redivivus* in alginate beads stored at 22^{0}C.

From these results (Table 7) it is shown that nematodes still persist in the capsules after 35 days at 22^{0}C. Survival was improved when stored in alginate beads compared to storage in foam chips, possibly due to the restricted movement preventing the use of energy reserves within the capsule. It therefore can be concluded that nematodes have a superior survival to nematodes stored in foam chips at 22^{0}C.

Table 7. Mean count of 4 replicates, Survival of *P. redivivus* in alginate beads stored at 22^{0}C.

Day	0	3	6	8	10
Mean of 4 replicates	211.00	181.50	152.00	152.00	181
Day	12	14	17	20	35
Mean of 4 replicates	137.00	100.25	70	77	7

Experiment 7

Survival of *P. redivivus* stored in alginate beads in concentrations of 10,000, 6000, 4000 and 2000 nematodes per bead at 5^{0}C.

Capsules containing 6000, 4000 and 2000 nematodes were not significantly different in their survival from each other throughout their experiment. However the treatment containing the highest concentration of nematodes remained significantly different at the 5% level from all the other treatments.

By chilling the reagents higher numbers of nematodes can be introduced into the capsules and stored at room temperature. Chilling effectively reduces the movement of the nematodes as they are formulated into the beads. Incorporating more than 6000 nematodes per bead decreased their survival in the capsule. The irregularity of the results obtained at the highest concentration treatment may be due to the formulation. At the highest concentration of nematodes formation of the beads becomes very irregular. Assessment of nematodes were from beads selected and not from the ratio of nematodes to alginate gel demonstrating that up to 6000 nematodes per capsule can be incorporated into alginate capsules and stored at room temperature for up to 30 days.

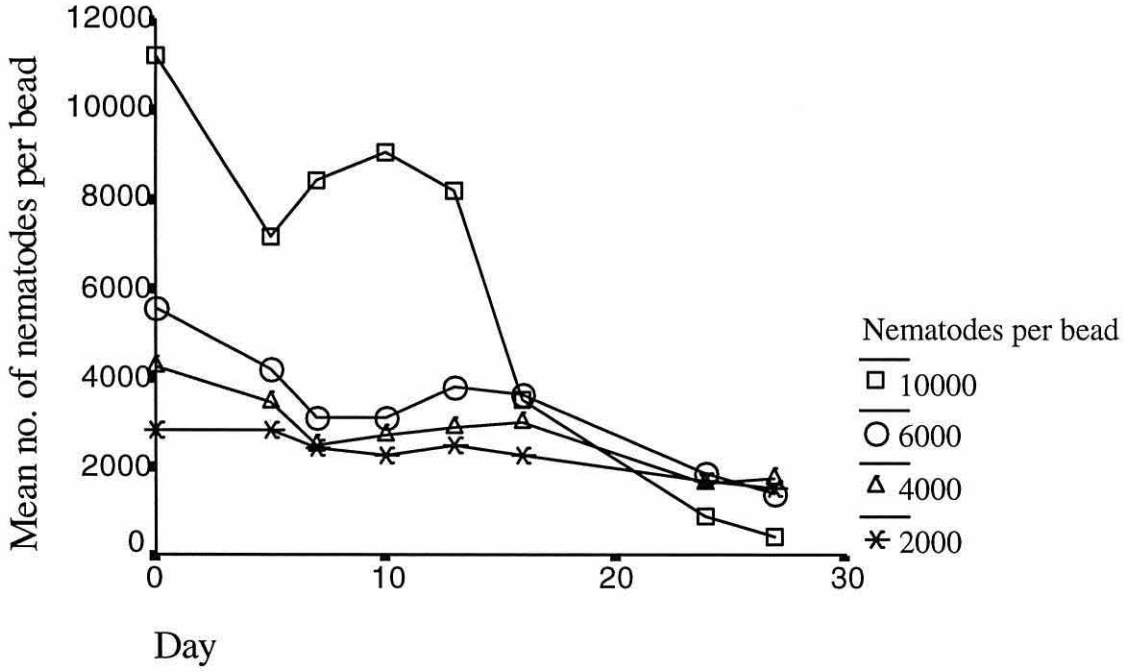


Figure 8. Survival of *P. redivivus* stored in alginate beads in concentrations of 10000, 6000, 4000 and 2000 nematodes per bead at 22^{0}C.

Table 8. Mean of 4 replicates, Survival of *P. redivivus* stored in alginate beads in concentrations of 10000, 6000, 4000 and 2000 nematodes per bead at 22^{0}C.

Treatments								
Day	0	5	7	10	13	16	24	27
2000	2850.00	2832.50	2407.50	2262.50	2485.00	2000.00	1677.50	1585.00
4000	4268.75	3443.75	2547.50	2603.50	2880.00	3250.00	1677.50	1585.00
6000	5081.75	4181.25	4181.25	3337.50	3800.00	3493.75	1865.00	1420.00
10000*	11187.50	7187.50	8450.00	4537.50	5869.50	3525.00	875.00	400.00

* significant

Experiment 8

Survival of *P. redivivus* stored in alginate beads in concentrations of 10,000, 6000, 4000 and 2000 nematodes per bead at 5^{0}C.

Table 8 shows that the treatment containing 10 000 nematodes stored in beads was significantly different from the other treatments at the 5% level. Treatments containing 6000, 4000 and 2000 nematodes per bead maintained their nematode numbers after 30 days. A decline in nematode numbers is clear in the highest concentration of nematodes used in beads. It can be concluded that the concentration of up to 6000 nematodes per capsule can be stored in alginate beads at 5^{0}C.

Table 9. Mean of 4 replicates, Survival of *P. redivivus* stored in alginate beads at 4 concentrations of 10 000, 6000, 4000 and 2000 at 5^{0}C.

Treatment					
Day	0	7	14	28	31
2000	2950.00	2875.00	2670.00	2579.50	2357.50
4000	4268.75	4187.50	3800.00	5031.25	3475.00
6000	5056.25	3756.25	4131.25	4987.50	4381.25
10000	11187.50	7807.50	9337.50	7481.25	4468.75

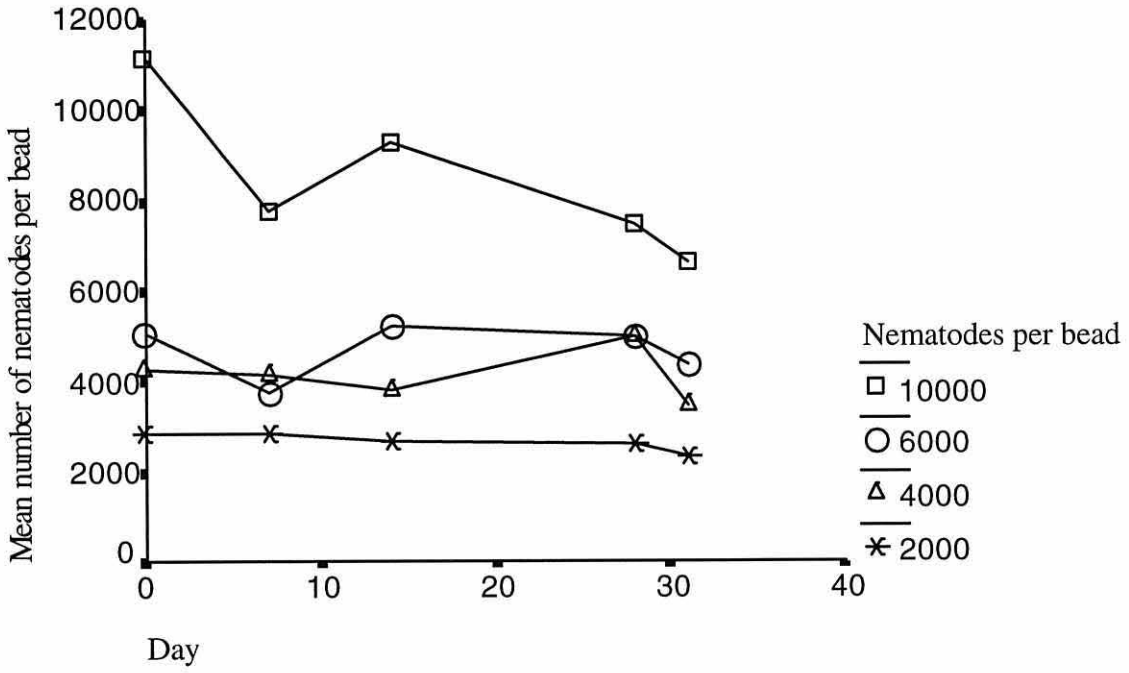


Figure 9. Survival of *P. redivivus* stored in alginate beads at 4 concentrations of 10 000, 6000, 4000 and 2000 at 5^0C.

With reference to the results (Table 9) the treatment containing 10 000 nematodes stored in beads was significantly different from the other treatments at the 5% level. Treatments containing 6000, 4000 and 2000 nematodes per bead maintained their nematode numbers after 30 days. A decline in nematode numbers is clear in the highest concentration of nematodes used in beads. It can be concluded that the concentration of up to 6000 nematodes per capsule can be stored in alginate beads at 5^0C.

Experiment 9

Survival of *P. redivivus* stored in alginate beads at 4 concentrations below 4000 nematodes per bead and stored at 5^0C, 18^0C and 22^0C.

Treatment **A** contains an average of 1163 nematodes per bead, Treatment **B** contains an average of 1688 nematodes per bead, Treatment **C** contains an average of 2038 nematodes per bead and

Treatment **D** contains an average of 3888 nematodes per bead.

Comparisons were made between the same treatments at different temperatures until day 54. No surviving nematodes were found beyond this day in treatments stored at 18^{0}C and 22^{0}C. At day 5 significant differences were observed between the same treatments stored at 22^{0}C, 18^{0}C and 5^{0}C at the 5% level. Significant differences were not observed between the same treatments at 18^{0}C and 5^{0}C. This significant difference was maintained until day 54 when only the treatments A and B remained for 22^{0}C and 18^{0}C. These were compared to the same treatments at 5^{0}C. It was found that 22^{0}C and 18^{0}C were not significantly different from each other but were significantly different at the 5% level from treatments stored at 5^{0}C. Nematodes stored in alginate beads at 5^{0}C survived longer than other treatments stored at 18^{0}C and 22^{0}C.

Table 10. Mean of 4 replicates, The survival of *P. redivivus* in alginate beads at 4 concentrations at 22^{0}C

Day	TreatmentA	TreatmentB	TreatmentC	TreatmentD
0.00	1163.00	1688.00	2038.00	3888.00
5.00	982.50	1290.00	2226.00	3440.00
13.00	1318.00	1470.00	2310.00	2008.00
32.00	670.00	605.00	0.00	0.00

Table 11. Mean of 4 replicates, The survival of *P. redivivus* in alginate beads at 4 concentrations at 18^{0}C

Day	TreatmentA	TreatmentB	TreatmentC	TreatmentD
0.00	1163.00	1688.00	2038.00	3888.00
5.00	1078.00	1668.00	3125.00	2458.00
13.00	1183.00	1628.00	2408.00	2615.00
32.00	893.00	1378.00	2553.00	1380.00
54.00	54.00	0.00	0.00	0.00

Table 12. Mean of 4 replicates, The survival of *P. redivivus* in alginate beads at 4 concentrations at 5^{0}C

Day	TreatmentA	TreatmentB	TreatmentC	TreatmentD
0	1163.00	1688.00	2038.00	3888.00
5	1093.00	1745.00	3850.00	4019.00
13	670.00	1220.00	2183.00	2688.00
32	1080.00	1423.00	2230.00	1436.00
39	902.00	1518.00	3294.00	2500.00
54	855.00	1625.00	2923.00	1919.00
64	900.00	1520.00	1208.00	3363.00
74	863.00	1198.00	2132.00	2388.00
80	905.00	1340.00	2613.00	3775.00
87	1197.00	1535.00	2869.00	643.00
94	815.00	1240.00	3031.00	938.00
108	833.00	1213.00	2065.00	0.00

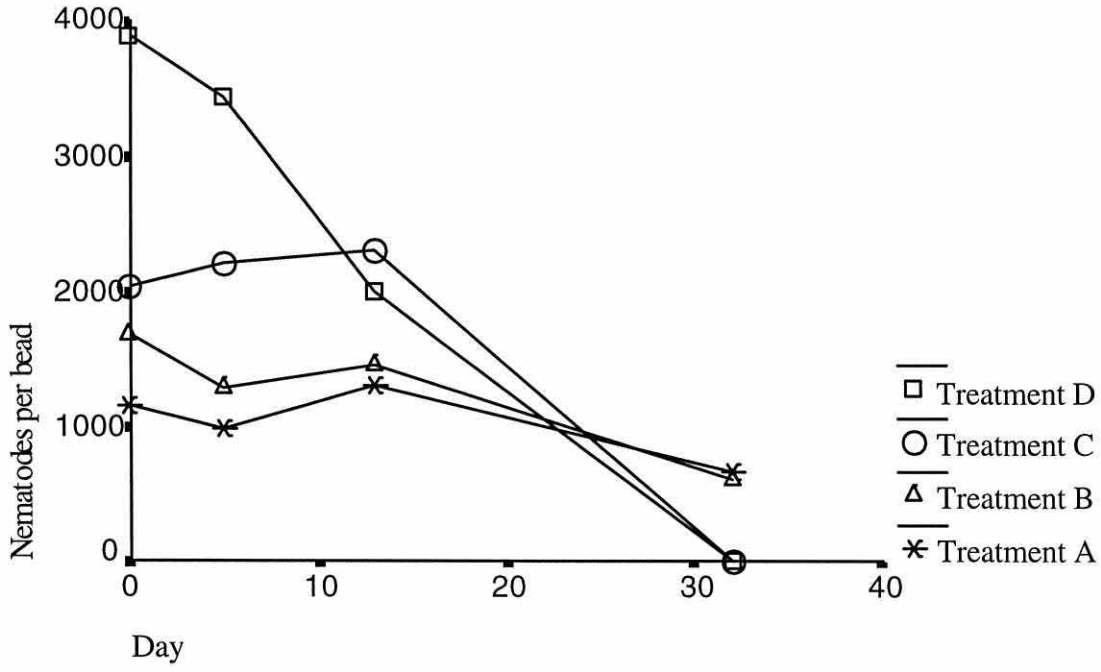


Figure 10. The survival of *P. redivivus* in alginate beads at 4 concentrations at 22^{0}C

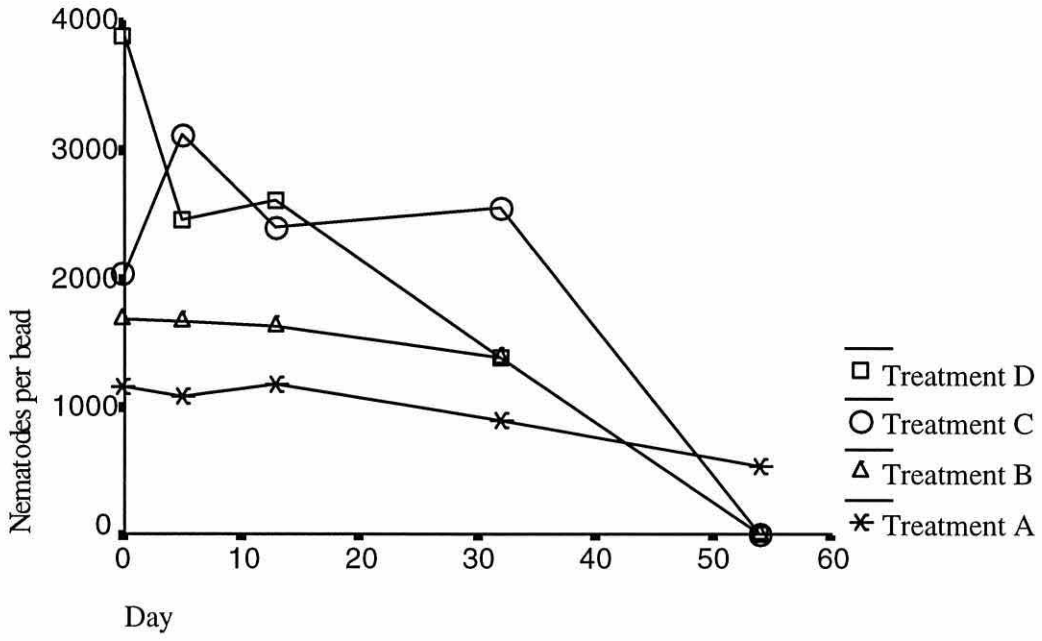


Figure 11. The survival of *P. redivivus* in alginate beads at 4 concentrations at 18^{0}C

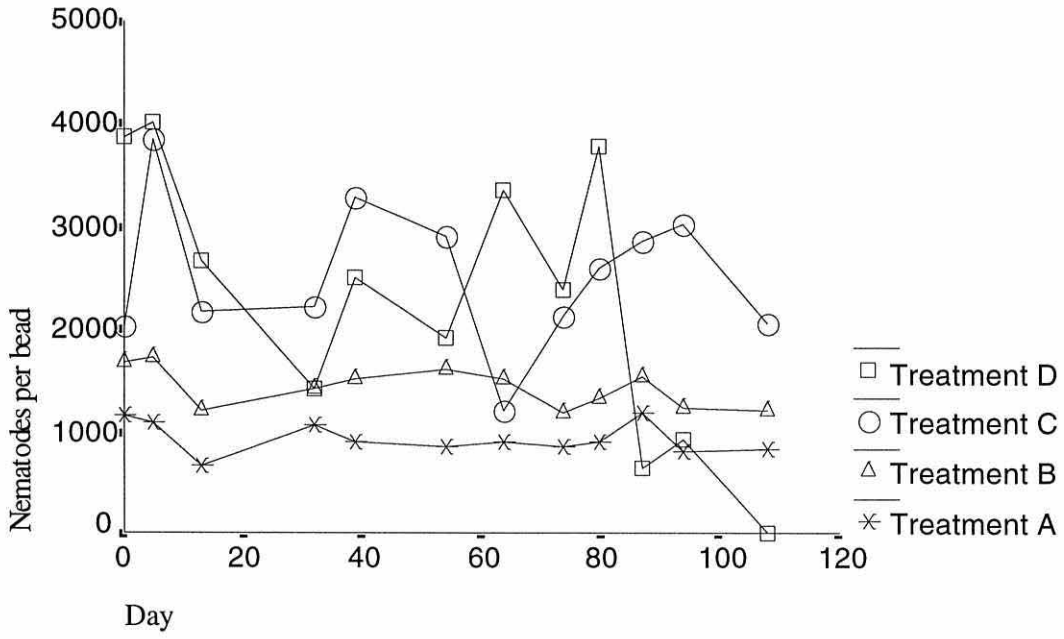


Figure 12 The survival of *P. redivivus* in alginate beads at 4 concentrations at 5^{0}C.

Experiment 10

Survival of P. redivivus stored in 50g bags of alginate beads.

Figure 13 shows the results of either a) counting nematodes extracted from one bead (an average taken from 4 beads) or b) nematodes extracted from a 10g of beads and then dividing by the number of beads in 10g to obtain the number of nematodes per bead, significant differences were observed between the two methods of extraction at the 5% level (Table 13) from day 68 onwards. Nematode survival was shown to decrease in samples taken by measuring individual beads, this was to be expected as individal beads tended to dessicate after storage for long periods of time. An increase in the numbers was observed for the method of measuring weight of alginate to nematode numbers extracted. It was concluded that measuring the same weight of alginate over a long period of time is inaccurate due to the water loss from the alginate gel and an apparent increase in nematode numbers is observed and reflected by the results. It was concluded that measuring nematode numbers by selecting individual beads was the appropriate method.

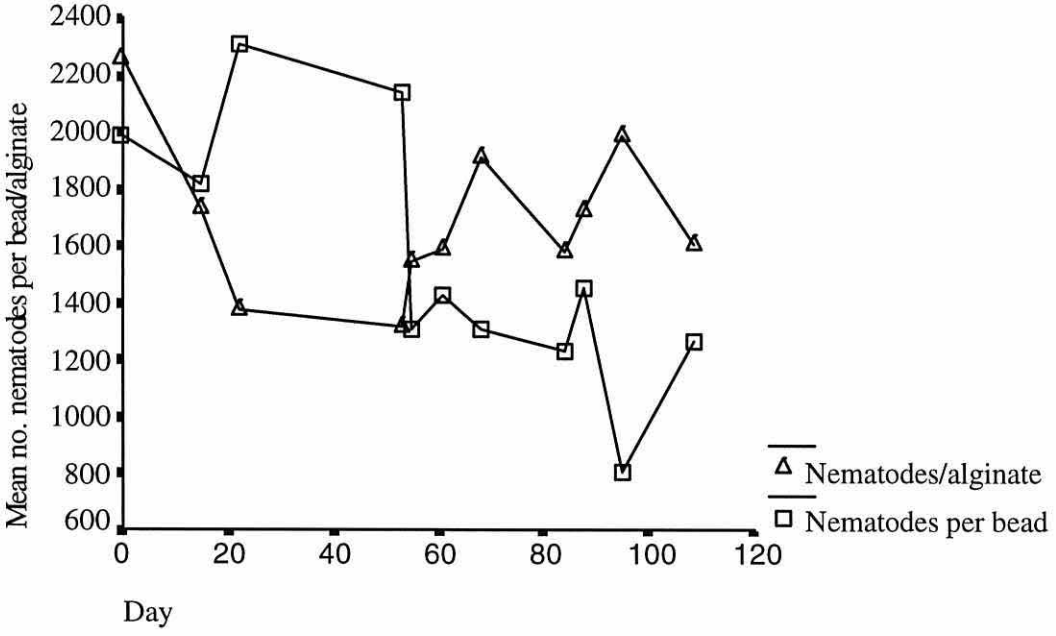


Figure 13. The survival of *P. redivivus* stored in 50g bags of alginate beads

Table 13. Mean of 4 replicates, The survival of *P. redivivus* stored in 50g bags of alginate beads

Day	0	15	22	53	55	61	68	84	88	95	109
Bead	2256	1730	1380	1318	1546	1590	1913	1580	1725	1988	1608
wt	1986	1816	2311	2142	1312	1432	1312	1229	1454	804	1266

Experiment 11

Loss of pigment from alginate beads stored at 22^{0}C.

In Table 14, pigment is lost from the guts of the nematodes rapidly when stored at room temperature. However the rate of loss of pigment slows down and becomes more stable after day 4 (Fig 14). This may be due to some astaxanthin being incorporated into the nematode tissues. It would be beneficial to observe loss of pigment from nematodes stored in alginate beads at cooler temperatures. Due to limited time and the removal of the spectrophotometer from the research laboratory this was not possible.

Table 14. Calculations of pigment present per gramme of dry weight of nematodes.

Day	Absorption	mg/L*	mg**	g/d.w.g***
0	2.82	0.026	0.00026	$1.721x10^{-6}$
1	2.73	0.025	0.00025	$1.655x10^{-6}$
2	1.10	0.010	0.00010	$1x10^{-6}$
3	0.86	0.0082	0.000082	$5.43x10^{-7}$
4	0.57	0.0056	0.000056	$3.7x10^{-7}$
5	0.62	0.0060	0.000060	$3.97x10^{-7}$
6	0.49	0.0049	0.000049	$3.24x10^{-7}$
7	0.73	0.0071	0.000071	$4.7x10^{-7}$

* Calculated from the absorption equation y=x108.43 - 0.0389.

** Amount of astaxanthin (mg) present in 10ml of acetone.

***Amount of astaxanthin (g) present in 1g dry weight of nematodes.

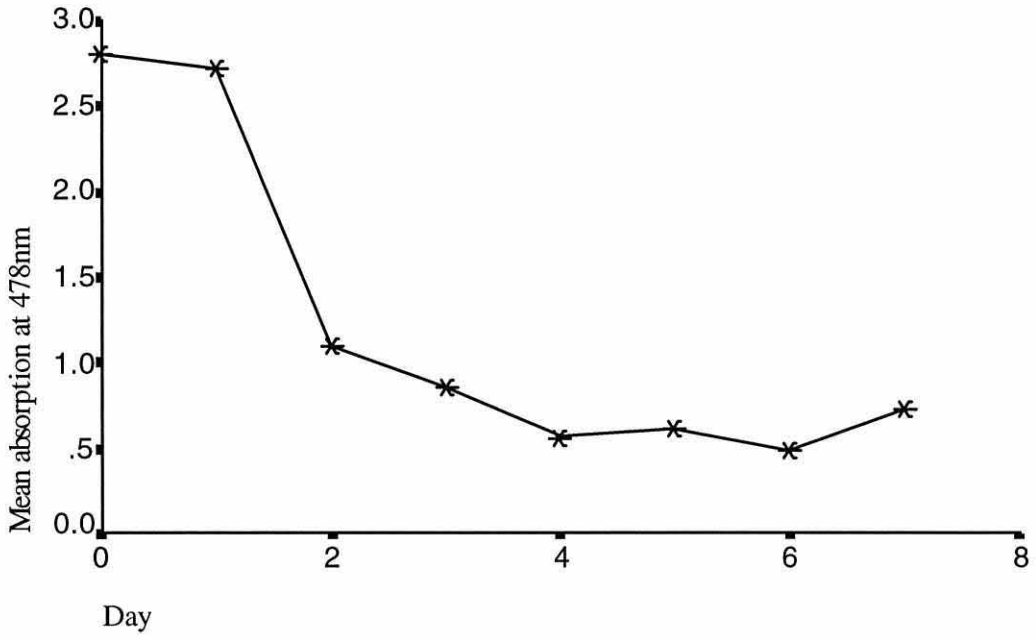


Figure 14. Loss of pigment from alginate beads stored at 22^{0}C

Experiment 12

Loss of pigment from *P. redivivus* stored in alginate beads and foam chips stored in bags.

The results (Table 15) indicate that nematodes stored in alginate beads at 5^{0}C retain the pigment longer than nematodes stored in foam chips in bags. This may be due to the restriction of nematode movement in the beads and hence the pigment is retained for a longer period of time in the gut. After day 5 of storage nematodes stored in alginate beads are significantly different at the 5% level from day 5 and remain so until the termination of the experiment.

Table 15. Mean of 4 replicates, Loss of pigment from *P. redivivus* nematodes stored in alginate beads and foam chips.

Day	Bags%	Beads%	Significant
0	76	80	Ns
2	71	76	Ns
3	73	76	Ns
5	52	67	*
8	47	70	*
10	38	65	*
12	22	64	*

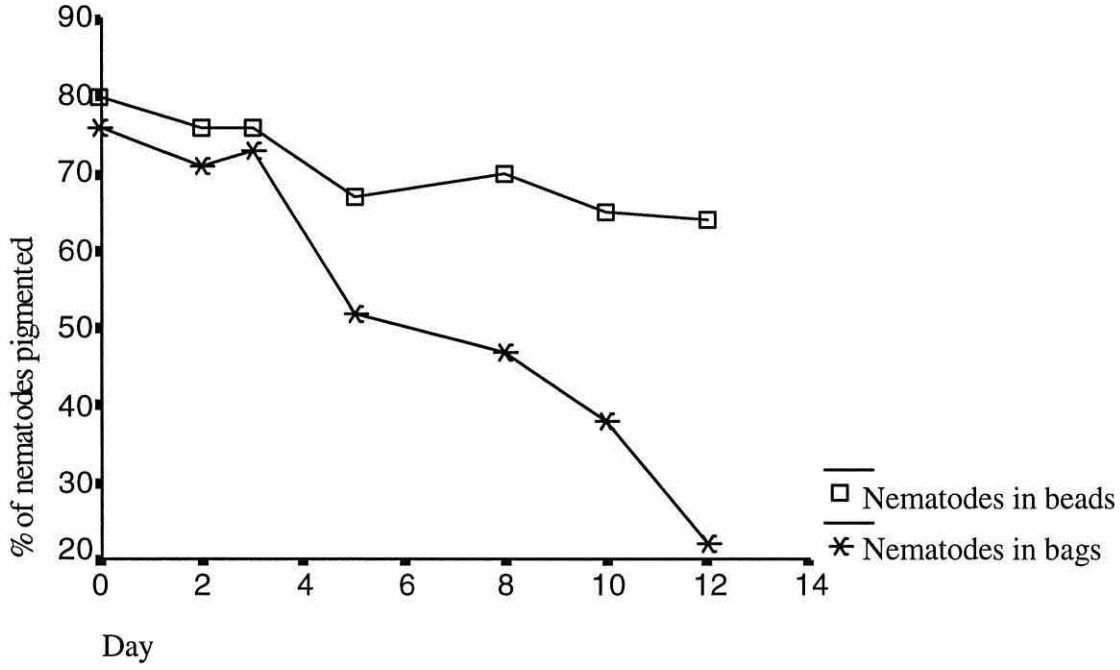


Figure 15. Loss of pigment from alginate nematodes stored in alginate beads and foam chips stored in bags.

DISCUSSION

Investigations into the use of clays and polyacrylamide gels were not undertaken due to the difficulty of extraction of the nematodes from these formulations and the requirement to use toxic chemicals to complex the gels. The use of polyethylene foam chips and alginate gel were investigated for the storage and easy extraction of *P. redivivus*. High temperature short term storage experiments in foam chips revealed that 5 and 10 million nematodes could be stored in 2g or 3g and 1g and 2g of foam chips respectively with either 15ml or 10ml of water. These conditions provided the optimum storage environment at 22^0C with good air circulation and moisture provided under these conditions. During a long term storage trial at 5^0C lipid and protein analysis were undertaken on the nematodes. These results showed that nematodes may be stored for up to 4 weeks under these conditions although chemical analysis of these nematodes showed an initial decline in total fatty acid this then remains relatively stable until week 10. There is a decrease in the fatty acids (14:0, 18:1ω-9, 18:4ω-3, 20:1ω-9, 22:6ω-3 and 22:1) and an increase was observed in (18:0, 20:4ω-6, 20:5ω-3). The percentage of saturated fatty acids increased and monounsaturated fatty acids decreased, with monosaturated decreases in 16:1ω-9, 18:1ω-9 and 20:1ω-9 and 22:1ω-11. The general increase in saturated fatty acids was due to an increase in 18:0. The overall total fatty acid content remains similar suggesting a conversion between saturated and monosaturated fatty acids or a depletion in the lower chain fatty acids which could not be detected with the equipment used. A possible energy source for the nematodes could be (18:1ω-9, 18:2ω-6, 18:3ω-3 and 18:4ω-3).

In an attempt to increase the survival of nematodes they were incorporated into alginate gel. The results of an initial experiment (fig.7.) indicates that nematodes survived for up to 35 days in alginate beads. Initial experiments demonstrated that a maximum of only 200 nematodes per bead can be incorporated (fig.8.). The increase in survival of the nematodes could be attributed to the prevention of movement of the nematodes reducing the use of valuable energy reserves within the capsule. By chilling the reagents and the nematode paste before the nematodes are incorporated into the beads it was shown that increased numbers of nematodes per bead could be incorporated (fig.9.). Good survival was observed for beads containing 6000, 4000 and 2000 nematodes per bead. Above these concentrations irregularities in bead formation were observed

and it was thought this may effect the accuracy of counts of nematodes obtained from the beads. This effect was examined in experiment 10, where a higher consistency was obtained with nematodes extracted from individually counted beads than from measure and weights of alginate gel. This was likely to be due to the loss of water from the gel over a long period of time thereby increasing the amount of nematodes extracted from the gel.

It was further established that survival of nematodes was superior in alginate beads compared to foam chips. When experiments were conducted to observe the loss of the pigment astaxanthin from nematodes stored in alginate beads (fig.14.) it was found that the loss of pigment from the nematodes is rapid at 22^{0}C. Rates of pigment loss in the nematodes stored in alginate beads as opposed to foam chips, revealed that pigment was maintained in the guts of the nematodes far longer in alginate gel. Sixty to seventy percent of nematodes in alginate beads contained pigment in their guts after 12 days at 22^{0}C, probably due to the restricted movement of the nematodes within the alginate beads and hence the retention of pigment for longer periods of time in the gut.

Overall results indicate that the optimum storage material for the nematode *P. redivivus* is alginate gel beads stored at low temperatures between 0-5^{0}C containing 2000 to 6000 nematodes per bead.

The technique for encapsulating entomopathogenic nematodes in alginate gels is thought to greatly increase the potential of survival of nematodes for use as biological control agents. Redenbaugh et al (1984) described a method using 2g Na alginate in 100ml of water in a commercial blender for 4-5 min after which the nematodes are placed in this solution at a known concentration and dripped into calcium chloride solution before complexing for 20 -30 min. The beads were then washed with de ionised water, each capsule was found to contain approximately 300 nematodes which accumulated towards the centre of the bead. A similar finding was demonstated when *P. redivivus* was complexed in alginate beads. Nematodes were observed to collect towards the centre of the beads, but the vigorous movement of the nematodes during this process caused damage to the nematodes. However cooling the complexing agents served to reduce the movement of the nematodes and to prevent the damage.

The overall survival of *P. redivivus* nematodes is much increased when formulated in alginate beads. Kaya and Nelsen (1985) encapsulated *S. feltiae* and *H. heliothidis* in calcium alginate gel. Nematodes survived for up to 5 months with no detectable decrease in infectivity or survivability at 4^0C. However the conditions in which nematode capsules are stored also affect the survival of the nematodes (Table 2).

Table 14. Behaviour of infective juveniles *S. feltiae* embedded in alginate capsules and held under various conditions. After Poinar et al (1985).

Condition	% Nematodes remaining in capsules after 7 days	State of nematodes remaining in capsules
Water (10^0C)	100	All living
Water (20^0C)	30	All living
Dry (20^0C)	23	All dead
Moist sterile sand (20^0C)	3	All living
Moist garden soil (20^0C)	2	All living
Nutrient agar (20^0C)	0	-
Water agar (20^0C)	2	All living

It was also suggested by Connick Jr. et al., (1991) that *S. carpocapsae* could be blended with moistened clay powder and ingredients that benefit the organisms. The mixture was kneaded and a thin sheet is rolled out and dried overnight at room temperature. The next day the mixture was ground up into granules. Nematodes are then released from the granules when they are moistened. These granules can be kept for up to 9 weeks in a refrigerator.

Other methods that could have been investigated include anhydrobiosis and cryopreservation. Barret (1989) developed desiccation procedures for *Ditylenchus spp*, *S. feltiae* and *Heterorhabditis spp*. Cryopreservation is a routine method for preserving nematode strains. Cryoprotectants such as methanol, ethandiol, glycerol and dimethyl sulfoxide are used. Anhydrobiosis is a term used for organisms capable of surviving with rapid or slow dehydration up to and beyond the point at which metabolism is fully arrested (Womersley, 1987). *S. carpocapsae* to a certain extent can be reduced to anhydrobiotic state (Popiel, 1987 unpublished

data). The ability to desiccate *P. redivivus* would be a step forward in providing a formulation that had a longer shelf life. Desiccated nematodes have an enhanced tolerance to extreme conditions as compared to non desiccated nematodes (Friedman, 1989 unpublished data). Experiments have shown that partially desiccated nematodes mixed with baits rehydrated inside the insect's body and penetrated into the body cavity and infected the host (Wojeik and Georgis, 1988; Georgis, 1987; cited in Georgis 1990). However for the use in the aquaculture or agrochemical industry the use of this method of storage is limited due to the fact that nematode quality degrades rapidly.

Future work.

Further investigation would need to be carried out on the alginate carrier other than the capsule form for optimum survival, for example producing alginate sheets holding a suspension of nematodes. Alginate sheets would perhaps allow larger volumes of nematodes to be incorporated into the matrix.

CHAPTER 5

PRELIMINARY SHRIMP LARVAL GROWTH TRIALS

Research for this chapter and chapter 6 was jointly conducted by myself who cultured and enriched the nematodes and Metin Kumlu who conducted the feeding trials. I took part in astaxanthin feeding trials with supervision by Dr. D. Fletcher and part of this work has been published as follows:

Fisher, C. M. and Rodgers, P.B. 1993. Lipid enhancement and pigmentation of the nematode *Panagrellus redivivus* for use as an aquaculture feed. *Proc. of the World Aquaculture Society, New Orleans, Louisiana, U.S.A. 14-18 Jan, 1993*. Abstracts, pp.237.

Fletcher, D.J., Fisher, C.M., Kumlu, M and Rodgers, P.B. 1993. Growth and survival of *Penaeus indicus* larvae fed on the nematode *Panagrellus redivivus. Proc. of the World Aquaculture Society, New Orleans, Louisiana, U.S.A. 14-18 Jan, 1993.* Abstracts, pp.109.

5. PRELIMINARY SHRIMP LARVAL GROWTH TRIALS

INTRODUCTION.

The major objective in penaeid aquaculture is to establish adequate feeding regimes that promote good growth and survival while being economical and practical. Under hatchery conditions current penaeid larval culture is still primarily dependent on live microalgal diets during protozoeal stages and *Artemia* nauplii during mysis and early postlarval stages (Hudinaga, 1942; Cook and Murphy, 1969; Emmerson, 1980; Liao, 1984). Problems encountered using algal cultures to rear penaeid larvae in large quantities have been well documented (Liao et al., 1983; Watanbe et al., 1983; Jones, 1988; Leger and Soorgeloos, 1992; Jones et al., 1993). Although *Artemia* is a very suitable seed source for the culture of *Macrobrachium rosenbergii* (Ling, 1969a, b; Deru, 1990), *Palaemon elegans* (Brewster, 1987), and mysis and early postlarval stages of penaeids (Sorgeloos 1980). The high price (Langdon et al., 1985) availability, nutritional quality and variations in hatching efficiency between different strains (Watanabe et al., 1983; Leger et al., 1986) are also well known drawbacks. In addition, *Artemia* consume algal feeds and grow quickly becoming unavailable as food for the penaeid larvae (Biedenbach et al., 1989). As a result, different alternative diets such as artificial microparticulated diets (Jones et al.,1979a; Jones et al., 1987; Galgani and Aquacop, 1988; Ottogali, 1991) and other live diets such as rotifers (Emmerson, 1984; Lovett and Felder, 1988) have been investigated as potential feeds to replace the conventional live diets used to rear commercially important shrimp larvae.

Free-living nematodes with their suitable size range, easy cultivation and high nutritional value were suggested as an important potential live food for rearing fish fry (Kahan et al., 1980). Samocha and Lewinsohn (1977) first reported successful use of the nematode, *Panagrellus sp.*, along with algae and *Artemia* nauplii, in rearing *Penaeus semisulcatus* and *Metapenaeus stebbingi*. Wilkenfeld et al., (1984) replaced live *Artemia* nauplii in culture of mysis stages of three penaeid species, *P. aztecus, P. vannamei* and *P. setiferus* using *Panagrellus redivivus*. They observed that these penaeid larvae were able to consume and survive on the single nematode diets from as early as PZI stage. However, these authors suggested the use of *P. redivivus* as a partial or complete *Artemia* replacement only during mysis stages to avoid reducing growth,

survival and delaying metamorphosis. Biedenbach et al., (1989) investigated the feeding level of *P. redivivus* stages in conjunction with algae from PZ2 to PL stages in *P. vannamei.* These authors obtained equal growth, survival and metamorphosis in comparison to *Artemia*/algae when they fed the larval of this penaeid species on the nematode/algae feeding regime.

The objectives of this chapter are, therefore, to investigate the possibility of complete replacement of both microalgae and *Artemia* nauplii with live nematodes (*Panagrellus redivivus* and *Caenorhabditis elgans* for the culture of the marine penaeid, *Penaeus indicus.* An adequate feeding level of the nematode *P. redivivus*, was investigated for protozoeal and mysis stages of *P. indicus.* Nematodes with different size ranges were fed to *P. indicus* larvae to determine the size effects of the nematodes on growth, survival and metamorphosis rate of the larvae from PZ1 to PL stages. Also , survival and growth of the larvae fed one meal and two meals of nematodes per day were compared separately during protozoeal and mysis stages.

MATERIALS AND METHODS

Rearing procedures for *Penaeus indicus* larvae.

For each experiment larvae were obtained from individual females held in 50 l spawning tanks at 28^0C and 33pp salinity. 50% of the sea water was changed after the spawning and irradiated with UV light to reduce bacterial contamination and cartridge filtered through 0.2µm to eliminate food sources from the larvae. Protozoea (PZ1) were given a 15 min bath in an antibiotic (furazolidone) at 0.2ppm before larvae were stocked in experimental 2 l round bottomed flasks incubated at 28^0C +/- 0.5^0C(thermostatically controlled) and containing a larval density of 100 larvae l^{-1}. Optimum predator prey interaction and O_2 circulation was maintained without damage to the prawn larvae by continuous aeration supplied via silicone rubber tubing with glass tubes at the tip. Complete water exchanges were performed every 2 days when the larvae were also counted and a sample 20 were measured in length and staged. 50% water changes were made on alternate days. Live food counts were performed on individual flasks and appropriate amounts of the feed were added to maintain specific feeding regimes detailed below. Samples of the

larvae were measured at the commencement of each trial. *Artemia* cysts supplied by (INVE AQUACULTURE), Belgium) were continously hatched at 26 -28^{0}C at 32ppt salinity during the experiment. The naulpii were separated from the shells of the cysts and counted to estimate their density. Salinity for each experiment was maintained at 25ppt (optimal to rear *P. indicus* larvae) for all experiments by mixing filtered (0.2µm) and UV sea water with distilled water. All nematode species were obtained from the Agricultural Genetics Company.

Experiment 1

***P. indicus* larvae (PZ2/PZ3) were fed on either *Artemia* or *P. redivivus*.**

P. indicus larvae (PZ1) were fed 50 cells μ^{-1} of *Tetraselmis chuii* (50%) and *Rhinomonas reticulata* (50%) plus Frippak microencapsulated diets (INVE AQUACULTURE, Belgium). When the larvae reached PZ2/PZ3 larvae were stocked at 30 larvae l^{-1} in 2 l experimental flasks to assess the survival and growth of the larvae fed on the nematode *P. redivivus* and *Artemia* nauplii.

Table 1 demonstrates the feeding regimes investigated. Algal feeds were fed to the larvae together with *P. redivivus* and *Artemia* until day 3 when the algal concentrates were halved. On day 4 *P. indicus* larvae were fed only on either *Artemia* or the nematode diet until the end of the experiment. Growth and survival data was analysed using ANOVA using SpSS following Bartlett's test for homogeniety of variances.

Table 1. Feeding regimes used to rear *P. indicus* larvae from stages PZ2/3 to postlarval stage (PL).

	Diet A		**Diet B**	
Days	Algae (cells μl^{-1})	Artemia (ml^{-1})	Algae (cells μl^{-1})	Nematode (ml^{-1})
0	50 cells	1.5	50 cells	10.0
1	50 cells	1.5	50 cells	10.0
2	50 cells	1.5	50 cells	10.0
3	25 cells	3.0	25 cells	15.0
4	-	5.0	-	15.0
5	-	5.0	-	15.0

Experiment 2

P. indicus larvae (PZ1) were fed 2 nematode species _P. redivivus_ and _C. elegans_

P. indicus larvae at stage PZ1 were fed 2 nematode species *P. redivivus* and *C. elegans* at a density of 25 nematodes l^{-1} to see if the larvae would survive on the two nematode species from the first feeding stage (PZ1). On alternate days larval growth and survival measurement were performed from replicates for each treatment. Control larvae were reared on a mixed algal diet of *T. chuii* (25 cells μl^{-1}) and *S. costatum* (35 cells μl^{-1}) until PZ3/M1 stage. At this stage the experiment was terminated due to the heavy mortalities observed with the larvae fed the nematodes.

Experiment 3

P. indicus larvae (PZ1) fed 4 different concentrations of the nematode _P. redivivus_ (30, 40, 50 and 60 ml^{-1}) .

This experiment investigated *P. indicus* larvae fed 4 different concentrations of the nematode *P. redivivus* (30, 40, 50 and 60 ml^{-1}) from stage PZ1 to assess adequate nematode feeding density in the absence of microalgae. The previous experiment collapsed possibly due to larvae not being fed the appropriate feeding level of nematodes. The larvae were stocked as previously described and fed live mixed algae *T. chuii* (25 cells μl^{-1})/*S. costatum* (35 cells μl^{-1}). Three replicates were used for each treatment. On alternate days larval growth in total length were measured when complete water changes were performed.

Experiment 4

Algae feeds stopped at M2 stage and lipid enriched _P. redivivus_ nematodes were offered to the larvae at 15, 30, 45 and 60 nematodes ml^{-1}.

Optimum feeding density was not established from experiment 3. In this experiment *P. indicus* larvae were reared to PZ2/PZ3 larvae (85% PZ2) and were stocked in 2 l flasks at a density of

75 l^{-1}. The controls were fed the algal cell mix as previously described until mysis 1 (M1). When newly hatched *Artemia* five ml^{-1} were added to the flasks along with algae until PL stages. Algal feeds were stopped at M2 stage and *P. redivivus* was offered to the larvae at 15, 30, 45 and 60 nematodes ml^{-1}. The nematodes used were lipid enriched (see chapter 2). Growth rates (day 1 to day 5) and survival rates (day 2 to day 5) of the larvae were compared using two way ANOVA with days as a covariate. Growth and survival at metamorphosis were compared using one way ANOVA with appropriate pairwise comparison tests (Tukey's and Scheffe's pairwise comparison test) following Bartlett's test for homogeneity of variances.

Experiment 5

***P. indicus* larvae (PZ1) optimum feeding density with nematodes by feeding the larvae once or twice a day.**

The objective of this experiment was to investigate optimum feeding density of nematodes by feeding the larvae once or twice a day. *P. indicus* larvae (PZ1) were stocked as described in experiment 1. The nematode *P. redivivus* had been lipid enriched and pigmented (as described in chapter 2 and 4) and was fed to the larvae at a rate of 30 and 50 nematodes ml^{-1} between PZ1 and PZ3/M1 stages. The larvae were fed between the hours of 9.00h and 11.00h and then again between 22.00h and 24.00h. The nematodes were introduced into the flasks at these times as either one meal or as two meals a day (15 and 25 nematodes ml^{-1}). Growth and survival were assessed and analysed as previously described in the previous experiment.

Experiment 6

***P. indicus* larvae (PZ3/M1) optimum feeding density with nematodes by feeding the larvae once or twice a day.**

When *P. indicus* larvae had reached PZ3/M1 from experiment 5 the larvae were removed and stocked into 2 l experimental flasks at 75 larvae l^{-1} in two replicates to assess the optimum feeding density of nematodes during mysis and early PL stages. Three concentrations of

nematodes were investigated (60, 80 and 100 ml^{-1}) and fed as two meals a day as described in experiment 5. However survival data during protozoeal stages (experiment 5) and mysis stages (experiment 6) did not fit in the GLM, hence were excluded in the statistical calculations. Growth data was assessed by two way ANOVA with days as a covariate between day 2 and day 6 (PZ1, PZ3/M1) and between day 6 and day 9 (M1-PL1).

Experiment 7

Lipid enriched nematodes of various sizes fed to *P. indicus* larvae to PL stages.

This experiment was designed to investigate the effects of size on nematodes *P. redivivus* and larval development. Small (529+/-226µm mean length) and large size nematodes (1016+/-222µm) were obtained from myself. The nematodes were lipid enriched as described in chapter 2 and pigmented (see chapter 3). The following regimes were followed:

1) Large nematodes fed throughout larval development to PL stage (Large).
2) Small nematodes fed throughout larval development to PL stage (Small).
3) Small nematodes fed until PZ3/M1 stage and then large nematodes until PL stage. (S/L).
4) Mixed sized nematodes fed throughout all larval stages (mixed).

For all treatments the nematode concentration (ml^{-1} day) was increased from 30 (PZ1/PZ3 stages) to 45 (M1/M2stages) and finally 60 (M3-PL stages) during larval culture of *P. indicus*. Three replicates were used for the above 4 treatments. Larvae were measured and counted each day when complete water changes were made. Larval growth and survival during protozoeal stages (PZ1-PZ3/M1) or (day 2 to day 6) and mysis stages (m1-PL or day 6 to day 9) were compared using two way ANOVA with days as a covariate. Larval growth and survival at M1 and PL stages were compared using the method described in experiment 1.

RESULTS.

Experiment 1

P. indicus larvae (PZ2/PZ3) were fed on either *Artemia* or *P. redivivus*.

The results shown in Table 2 demonstrate that larvae reared on the nematode *P. redivivus* showed normal growth, development and survival through PZ2/3 and mysis stages to post larvae (see figures 1a and 1b). Nematodes promoted a higher survival (56%) and growth (5.7 mm total length) than those fed *Artemia* (38%, 5.5mm). However these differences were not significant at the 5% probability level (Tables 3a and 3b). The PLs fed nematodes were paler in colouration than *Artemia* fed larvae but equally healthy and active. Throughout the trial the larvae fed nematodes produced faecal strings that clearly demonstrated nematodes were being digested. By day six 75% of the larvae had attained the PL1 stage on the nematode diet (A) while only 60% of these reared on *Artemia* reached PL1 stage (Diet B).

Table 2. Growth and Survival data for figures 1a and 1b. Each value is a mean +/- s.d. (n=2). Diet A (algae/nematode) and Diet B (algae/*Artemia*).

	Diet A			Diet B		
Days	Stage	Survival (%)	Total length (mm)	Stage	Survival (%)	Total length (mm)
0		100.00	2.49 +/- 0.40		100.00	2.49 +/- 0.40
2		80.00 +/- 18.86	3.46 +/- 0.34		53.33 +/- 7.07	3.21 +/- 0.02
4		61.65 +/- 14.19	4.47 +/- 0.36		45.82 +/- 1.20	4.31 +/- 0.14
6	25%M3 75%PL1	55.83 +/- 9.17	5.69 +/- 0.02	40%M3 60%PL1	38.33 +/- 6.67	5.54+/-0.14

Table 3a. One-way ANOVA on larval survival (%) of *P. indicus* reared on the nematodes and a control diet from PZ2/3 to PL stages.

Source	DF	SS	MS	F	P	Significant
Diets	1	306.3	306.3	4.18	0.177	Ns
Error	2	146.4	73.2			
Total	3	452.7				

Bartlett's test for homogeneity of variances F = 0.119, P = 0.91

Table 3b. One-way ANOVA on larval growth (mm TL) of *P. indicus* reared on the nematodes and a control diet from PZ2/3 to PL stages.

Source	DF	SS	MS	F	P	Significant
Diets	1	0.044	0.044	0.10	0.754	Ns
Error	2	16.250	0.439			
Total	3	16.2924				

Bartlett's test for homogeneity of variances F = 2.514, P = 0.08

Experiment 2

P. indicus larvae (PZ1) were fed 2 nematode species *P. redivivus* and *C. elegans*.

C. elegans did not support survival and growth beyond day 3. *C. elegans* caused a high mortality as early as day 2 of the experiment resulting in 66% survival due to the contamination of the nematode cultures. I was unable to supply fresh nematodes for this trial and those that were used were not thoroughly cleaned from the medium and hence some of the culture medium entered the larval culture flasks. On day 2 the survival of the larvae was still 87%. This resulted in the immediate fouling of the shrimp larvae preventing them from feeding and moulting. As a result only 10% of the larvae moulted to M1 after this cultures collapsed. Mean survival and total length (mm) of control larvae was 80.5% and 3.98 mm (all M1 stage).

Experiment 3

P. indicus larvae (PZ1) fed 4 different concentrations of the nematode *P. redivivus* (30, 40, 50 and 60 ml^{-1}) .

The results in Table 4 demonstrate that the larvae consumed the nematodes and displayed very high survival and growth during early days of culture. At PZ3 the larvae weakened and a small quantity developed into M1. It was observed that the culture was fouled by the introduction of nematode culture medium and therefore the experiment was terminated at PZ3/M1.

Table 4. Larval survival (%) and growth (mm) of *P. indicus* fed on nematodes at densities from 30 to 60 ml^{-1} from PZ1 to PZ3/M1 stages. Each value is a mean +/- s.d. (n=3).

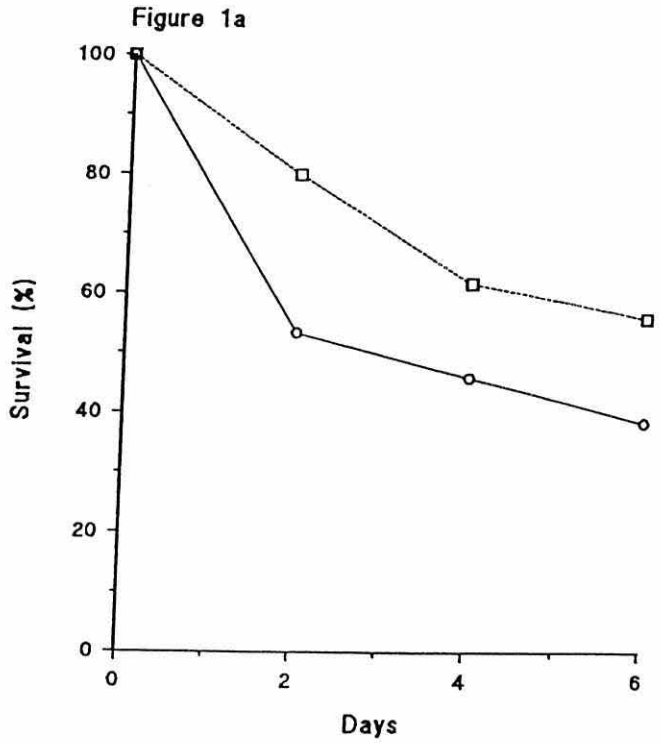
Survival (%)					
Days	30 nematodes (ml^{-1})	40 nematodes	50 nematodes	60 nematodes	Controls
0	100.00	100.00	100.00	100.00	100.00
2	75.55 +/- 2.78	77.50 +/- 6.00	82.50 +/- 2.65	78.50 +/- 4.27	89.67 +/- 7.29
4	38.17 +/- 4.31	31.00 +/- 2.46	28.67 +/- 5.06	23.33 +/- 3.88	51.17 +/- 4.31
Growth (mm)					
0	1.19 +/- 0.03	1.19 +/- 0.03	1.19 +/- 0.03	1.19 +/- 0.03	1.19 +/- 0.03
2	2.31 +/- 0.02	2.22 +/- 0.21	2.23 +/- 0.03	2.19 +/- 0.02	2.20 +/- 0.03
4	3.25 +/- 0.07	3.27 +/- 0.03	3.16 +/- 0.04	3.32 +/- 0.05	3.54 +/- 0.01

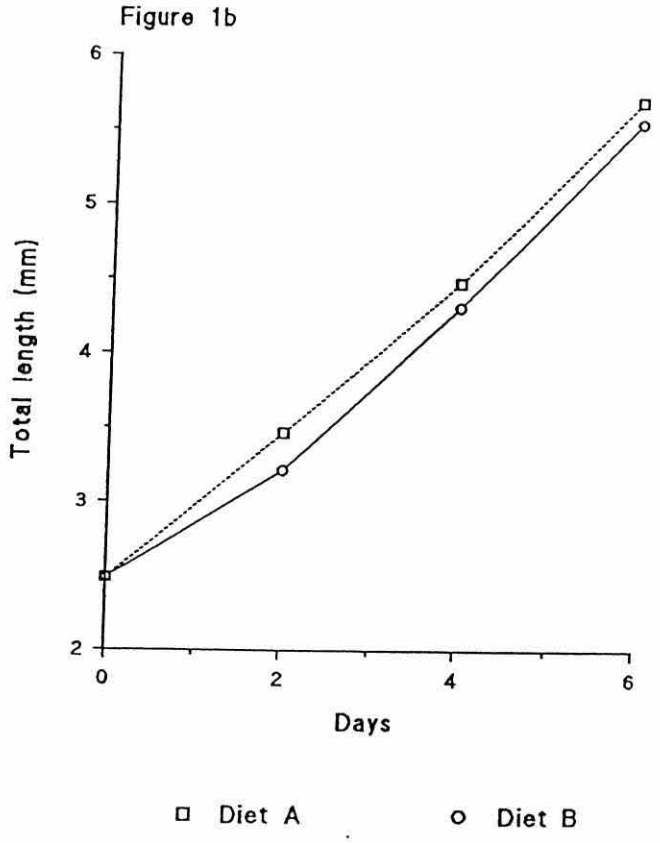
Experiment 4

Algae feeds stopped at M2 stage and lipid eniched *P. redivivus* nematodes were offered to the larvae at 15, 30, 45 and 60 nematodes ml^{-1}.

Table 5 summarises the growth and survival data. Figures 2a and 2b demonstrate that the nematode *P. redivivus* was a suitable substitute for *Artemia* nauplii as food for *P. indicus* larvae from stages PZ2/3 to PL1. Nematode fed larvae demonstrated normal growth and survival from PZ2/PZ3 and mysis stages to post larvae in the trial. Results in Tables 6 and 7a show that survival rate of the larvae fed on the nematode concentration (15-60 ml^{-1}) were not significantly different at the 5% probability level from each other.

Survival at the PL1 stage showed that the control diet gave significantly lower survival (66%) compared to all the other nematode diets which ranged from 83% to 89% (Table 5). The mortality rates for all nematode diets was significantly lower than the control diet (2.76% day^{-1}). The mean growth was highest for the feeding density of 60 nematodes ml^{-1} and the highest larval growth rate for the nematode treatments (0.526) was also achieved at this density. However there





Figures 1a and 1b. The survival (%) and growth (mm) of *P. indicus* larvae fed on Diet A (algae/nematode) and Diet B (algae/*Artemia*) from PZ2/PZ3 to PL stages. All values are means of two replicates.

was no significant difference between the treatments (30, 45 and 60 nematodes ml^{-1}) at the 5% probability level.

Higher concentrations of nematodes (45 and 60 nematodes ml^{-1}) produced a higher metamorphosis rate (PL1 87%) and (PL1 78%, PL2 10%) respectively compared to the lower treatments (15 and 30 nematodes ml^{-1}). This result suggests that a faster larval development is occurring for the highest concentration of nematode treatments. Larvae fed higher nematode concentrations (45 and 60 ml^{-1}) attained PL stages one day earlier than the lower concentrations (15 and 30 ml^{-1}). It can be concluded that the best feeding levels were obtained with 45 and 60 nematodes ml^{-1} throughout the experiment.

Table 5. The survival rate (% day^{-1}), mortality rate (% day^{-1}), growth at PL1(mm), growth rate (mm day^{-1}) and larval stage composition of *P. indicus* larvae reared on different densities of nematodes (ml^{-1}) and a control diet.

Diets					
	15 nematodes ml^{-1}	30 nematodes ml^{-1}	45 nematodes ml^{-1}	60nematodes ml^{-1}	Control
Survival at PL1 (%)	88.2+/-4.69	82.67+/-5.29	89.34+/-1.34	88.67+/-2.91	65.78+/-4.91
Mortality rate (% day^{-1})	0.850	1.443	0.517	1.023	2.739
Growth at PL1 (mm)	4.44+/-0.02	4.72+/-0.03	4.91+/-0.05	5.15+/-0.03	5.03+/-0.04
Growth rate (mm day^{-1})	0.463	0.486	0.500	0.526	0.534
Larval stages at PL (%)	54%M2, 20%M3, 26% PL	6%M2, 57%M3, 37%PL1	13%M3,87%PL1	12%M3,78%PL1, 10%PL2	36%M3,61%PL1, 3%PL2

Table 6. Data for figures 2a, b. each value represents a mean +/- s.d. (n=3).

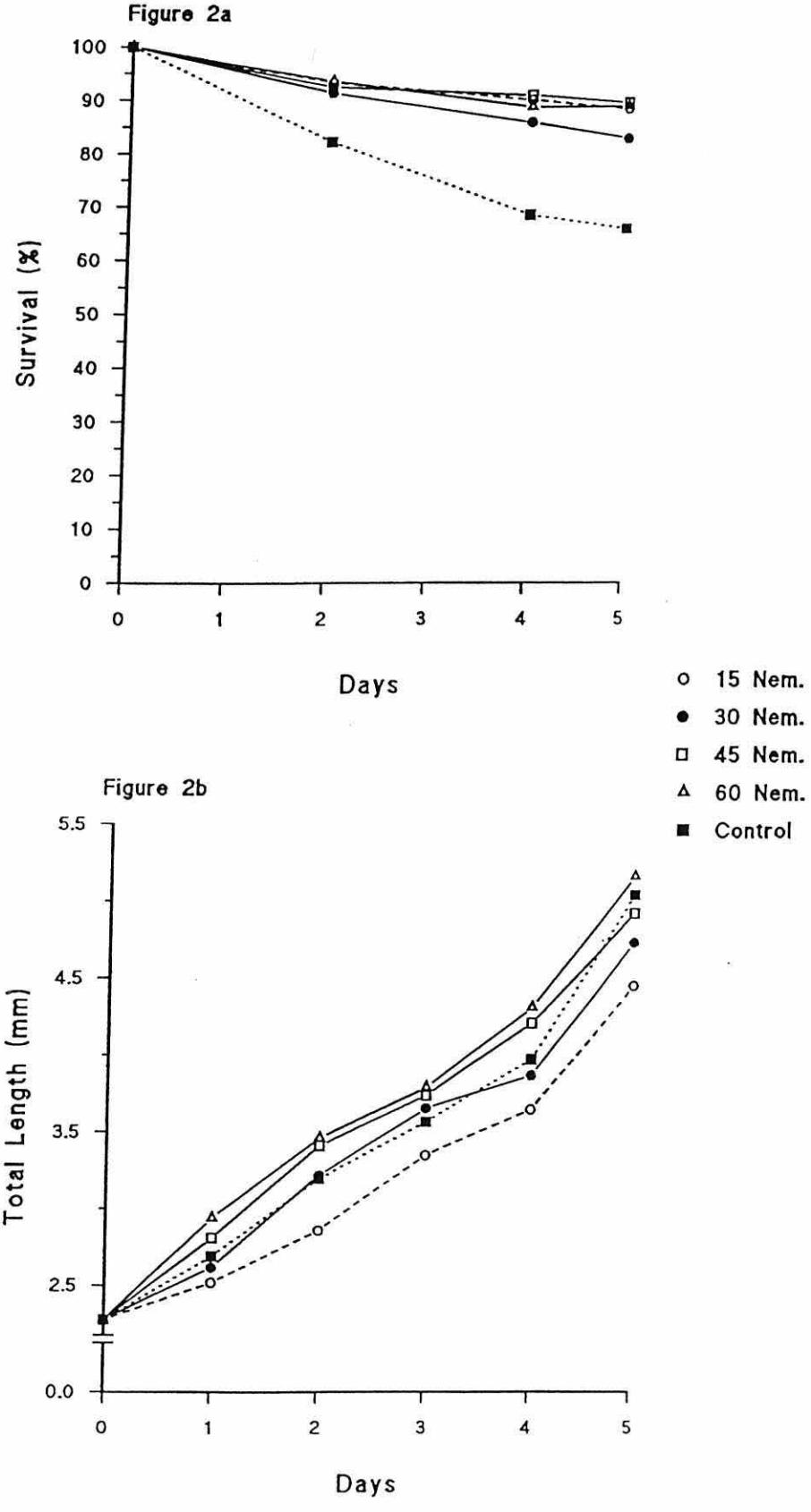
Larval survival (%) +/- s.d.					
Days	15 nem ml^{-1}	30 nem ml^{-1}	45 nem ml^{-1}	60 nem ml^{-1}	Control
0	100.00	100.00	100.00	100.00	100.00
2	93.33+/-4.00	91.33+/-3.34	92.45+/-2.34	93.56+/-6.05	82.22 +/-3.67
4	90.00+/-2.406	85.78+/-5.98	90.89+/-0.77	88.67+/-3.34	68.44+/-6.19
5	88.22+/-4.69	82.67+/-5.29	89.34+/-1.34	88.67+/-2.91	65.78+/-4.91
Larval growth (mm) +/- s.d.					
0	2.28 +/- 0.02	2.28 +/- 0.02	2.28 +/- 0.02	2.28 +/- 0.02	2.28+/-0.02
1	2.52 +/- 0.08	2.62 +/- 0.10	2.81 +/- 0.06	2.94 +/- 0.13	2.69 +/- 0.05
2	2.86 +/- 0.07	3.21 +/- 0.09	3.41 +/- 0.11	3.46 +/- 0.01	3.19 +/- 0.08
3	3.34 +/- 0.04	3.65 +/- 0.03	3.73 +/- 0.11	3.78 +/- 0.02	3.56 +/-0.07
4	3.64 +/- 0.14	3.86 +/- 0.04	4.20 +/- 0.18	4.30 +/- 0.12	3.97 +/- 0.05
5	4.44 +/- 0.08	4.72 +/- 0.06	4.91 +/- 0.10	5.15 +/- 0.08	5.03 +/- 0.06

Table 7a. Two-way ANOVA with days as a covariate on larval survival of *P. indicus* fed on various density of nematodes from PZ2/3 to PL1

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	4	2264.18	48.25	12.06	0.85	0.517	
Days	1	467.65	467.65	467.65	32.83	0.000	
Repl (Diets)	10	249.40	137.56	13.76	0.97	0.508	
Diets* Days	4	161.05	161.05	40.26	2.83	0.062	
Repl* Days (Diets)	10	116.05	116.21	11.62	0.82	0.619	Ns
Error	15	213.64	213.64	14.24			
Total	44	3472.12					

Table 7b. Two-way ANOVA, with days as a covariate, on larval growth of *P. indicus* fed on various density of nematodes from PZ2/3 to PL1.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	1	376.940	377.596	377.596	3999.20	0.000	
Days	4	27.898	2.807	0.702	7.43	0.000	
Repl (Diets)	10	0.810	0.779	0.078	0.83	0.604	
Diets* Days	4	1.000	1.006	0.252	2.66	0.032	
Repl* Days (Diets)	10	1.166	1.166	0.117	1.23	0.265	*
Error	720	67.981	67.981	0.094			
Total	749	475.796					



Figures 2a and 2b. The survival (%) and growth (mm) of *P. indicus* larvae fed various nematode concentrations (per ml) from PZ2/PZ3 to PL stages. All values are means of two replicates.

Table 7c. Comparison of the mortality rate (% day^{-1}) of *P. indicus* larvae fed on various densities of nematodes ml^{-1} from PZ2/PZ3 to PL stages.

Term	Mortality rate (% day^{-1})	Coeff.	Stdev.	t-value	P	Significant
Constant			93.901	1.488	0.000	
Days			-3.943	0.689	0.000	
Days*Diets						
15 nem	1.699	1.394	1.378	1.01	0.328	Ns
30 nem	2.885	-0.385	1.378	-0.28	0.784	Ns
45 nem	1.034	2.392	1.378	1.74	0.103	Ns
60 nem,	2.046	0.874	1.378	0.63	0.536	Ns
Control	5.478	-4.274	1.378	-3.10	0.007	**

Table 7d. Comparison of larval growth rates (mm day^{-1}) of *P. indicus* fed on various concentrations of nematodes (ml^{-1}) from PZ2/Z3 to PL stages.

Term	Growth rate (% day^{-1})	Coeff.	Stdev.	t-value	P	Significant
Constant		2.16935	0.02632	82.44	0.000	
Days		0.5011808	0.00793	63.24	0.000	
Days*Diets						
15 nem	0.463	-0.03873	0.01579	-2.45	0.014	*
30 nem	0.486	-0.01563	0.01587	-0.99	0.325	Ns
45 nem	0.500	-0.00175	0.01587	-0.11	0.912	Ns
60 nem,	0.526	0.02439	0.01587	1.54	0.125	Ns
Control	0.534	0.03172	0.01595	1.99	0.047	*

Experiment 5

P. indicus larvae (PZ1) optimum feeding density with nematodes by feeding the larvae once or twice a day.

Figure 3a and Table 9a show the survival of larvae fed various feeding regimes between PZ1 and PZ3/M1 stages. Survival data did not fit into the GLM and therefore statistical analysis could not be performed. During the first 3 days of culture nematodes supported high survivals (99-100%). On day 4 larvae fed once a day with 30 and 50 nematodes ml^{-1} showed higher mortalities compared to larvae fed twice a day and appeared dirty. The cultures became fouled between day 4 and 6. The experiment was terminated on day 6 and larvae fed 30 nematodes ml^{-1} twice a day showed the highest survival (71%) compared to the other feeding regimes. Lowest survival was observed in larvae fed once a day at 30 nematodes ml^{-1} (21.17%) and 50 nematodes ml^{-1} (52.67%) (Table 9a). Feeding the larvae twice a day with 50 nematodes ml^{-1} (25 nematodes ml^{-1}) twice daily was not successful.

Table 8a shows that larval growth rate was significantly affected at the 1 % level by nematode feeds during protozoeal stages. The highest larval growth rates were obtained for larvae reared at 30 nematodes ml^{-1} in two meals (0.667 m day^{-1}) and 50 nematodes ml^{-1} once a day (0.671 mm). Results in Table 8b show that larvae fed 30 nematodes ml^{-1} once a day showed significantly lower growth rate (0.566 mm day^{-1}).

Table 8a Two-way ANOVA with days as a covariate on growth of *P. indicus* larvae from PZ1 to PZ3/M1 (day 2 to day 6) on various feeding regimes.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	3	5.375	0.485	0.162	2.11	0.097	
Days	1	521.453	520.449	520.449	6803.36	0.000	
Repl (Diets)	8	0.796	0.548	0.069	0.90	0.520	
Diets* Days	3	2.319	2.335	0.778	10.17	0.00	
Repl *Days (Diets)	8	0.678	0.678	0.085	1.11	0.356	
Error	634	48.500	48.500	0.076			***
Total	657	579.121					

Table 8b. Comparisons of larval growth rates (mm day^{-1}) from PZ1-PZ3/M1 stages.

Term	Growth rate (mm day^{-1})	Coeff	St dev	T-value	P	Significant
Constant		0.89218	0.02558	34.88	0.000	
Days		0.631897	0.00766	82.48	0.000	
Days*Diets						
30 nema x1	0.566	-0.06597	0.01331	-4.96	0.000	***
30 nema x2	0.667	0.03498	0.01325	2.64	0.008	**
50 nemax1	0.671	0.03954	0.01331	2.97	0.003	**
50 nema x2	0.623	-0.00855	0.01321	-0.65	0.518	Ns

Table 9a. Survival data (%) for Figure 3a. each value is mean+/- s. D. (n=3).

Days	30 nema x 1	30 nema x 2	50 nema x 1	50 nema x 2
1	100.00	100.00	100.00	100.00
2	100.00	99.83 +/- 0.29	100.00	100.00
3	100.00	98.67 +/- 2.31	100.00	100.00
4	79.00+/-13.94	97.00 +/- 3.28	84.83 +/- 6.03	96.00+/-4.33
5	30.67 +/- 9.65	79.00 +/- 16.71	48.47 +/- 29.75	68.33+/-22.99
6	21.17 +/- 5.20	71.17 +/- 15.33	38.00 +/- 25.64	52.67 +/- 29.61

Table 9b. Growth (mm) data for figure 3b. Each value ia a mean +/- s. d. (n=3).

Days	30 nema x 1	30 nema x 2	50 nema x 1	50 nema x 2
1	1.12+/-0.02	1.12+/-0.02	1.12+/-0.02	1.12+/-0.02
2	1.34+/-0.02	1.37+/-0.01	1.34+/-0.08	1.37+/-0.01
3	2.28+/-0.04	2.28+/-0.04	2.29+/-0.04	2.30+/-4.0
4	2.61+/-0.15	3.01+/-0.07	2.96+/-0.17	3.13+/-0.13
5	3.36+/-0.08	3.45+/-0.12	3.36+/-0.02	3.40+/-0.03
6	3.64+/-0.15	4.12+/-0.10	4.16+/-0.10	3.94+/-0.18

Figure 3a.

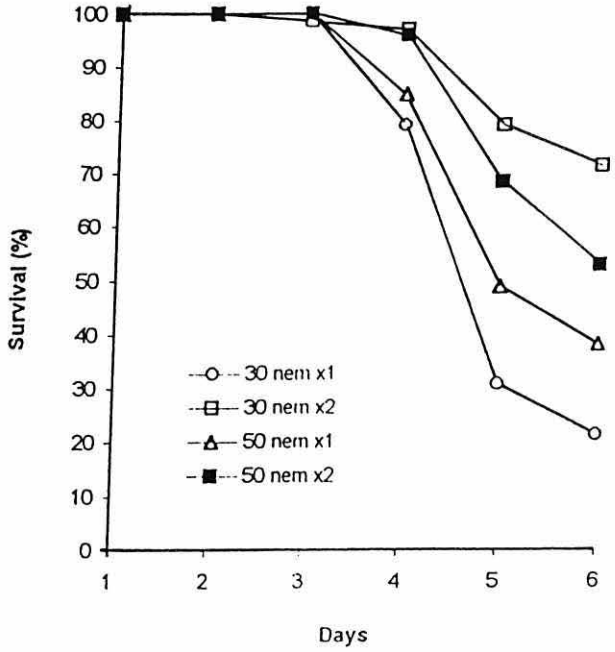
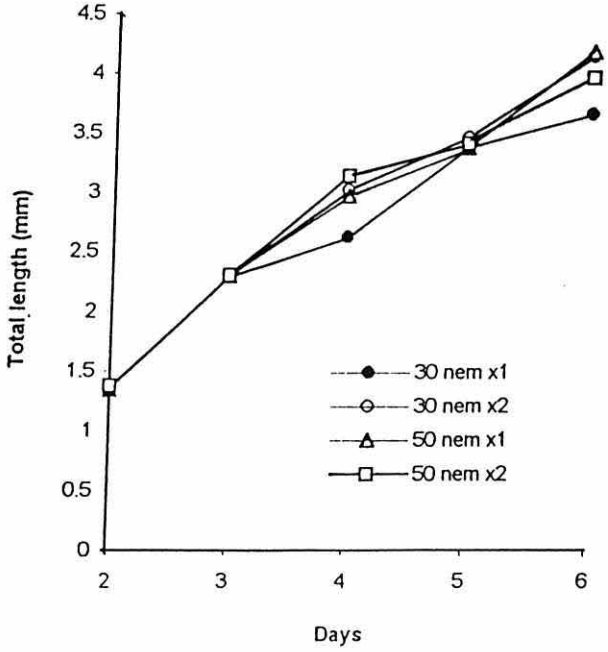


Figure 3b.



Figures 3a and 3b. The survival (%) and growth (mm) of *P. indicus* larvae fed on 30 or 50 nematodes ml^{-1} (x1) or two distribution times (x2) a day from PZ1 toPZ3/M1 stages. All values are means of two replicates.

Experiment 6

P. indicus larvae (PZ3/M1) optimum feeding density with nematodes by feeding the larvae once or twice a day.

The larvae fed various densities of nematodes at M1 showed a decline in survival over the first 2 days of the culture (Table 11). Larvae fed 60 nematodes ml^{-1} twice daily consistently displayed higher survival compared to those fed higher nematode concentrations. Due to problems in transport the nematode culture had remained at room temperature for two days and it is thought that the larvae may have have been fouled by the introduction of contaminating bacteria with the nematodes.

There was no significant difference at the 5% level in the growth of larvae fed 60, 80 and 100 nematodes ml^{-1} fed twice daily (Fig.4b). The results in Table 11 show that the total lengths at metamorphosis were 5.29, 5.26 and 5.09 mm respectively at 60, 80 and 100 nematodes ml^{-1} and larval growth rates on these levels were 0.405, 0.418 and 0.446 m day^{-1} respectively.

Table 10a. Two-way ANOVA with days as a covariate on growth of *P. indicus* larvae from M1-PL1 (day 6 to day 9) on various feeding regimes.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	2	1.7571	0.4950	0.2475	2.46	0.088	Ns
Days	1	25.3691	25.3067	25.3067	251.12	0.000	
Repl (Deits)	3	0.3675	0.1827	0.0609	0.60	0.613	
Diets* Days	2	0.0398	0.0414	0.0207	0.21	0.814	
Repl *Days (Diets)	3	0.4830	0.4830	0.1610	1.60	0.191	
Error	201	20.2559	20.2559	0.1008			
Total	212	48.2725					

Table 10b. Comparisons of larval growth rates (mm day^{-1}) from M1 to PL1 stages.

Term	Growth rate (mm day^{-1})	Coeff	Stdev	t-value	P	Significant
Constant		3.89558	0.05717	68.14	0.000	
Days		0.42311	0.02670	15.85	0.000	
Days* Diets						
60 nem x2	0.405	-0.01825	0.03761	-0.49	0.628	Ns
80 nem x2	0.418	-0.00503	0.03743	-0.13	0.893	Ns
100 nem x2	0.446	0.02328	0.03824	0.61	0.543	Ns

Table 11. Survival (%) and growth data (mm TL) for figures 4a and 4b. each value is a mean +/- s.d. (n=2).

	Survival (%) +/- s.d.			Total length (mm) +/- s.d.		
Days	60 nema x 2	80 nema x 2	100 nema x 2	60 nema x 2	80 nema x 2	100 nema x 2
6	100.00	100.00	100.00	3.85+/-0.03	3.85+/-0.03	3.85+/-0.03
7	94.33+/-8.01	90.33+/-8.01	89.00+/-6.12	4.48+/-0.05	4.42+/-0.02	4.19+/-0.05
8	82.67+/-1.89	64.00+/-5.66	71.34+/-9.43	4.80+/-0.13	4.52+/-0.01	4.60+/-0.00
9	21.34+/-7.54	17.33+/-0.94	17.33+/-5.66	5.29+/-0.18	5.26+/-0.16	5.09+/-0.04

Figure 4a.

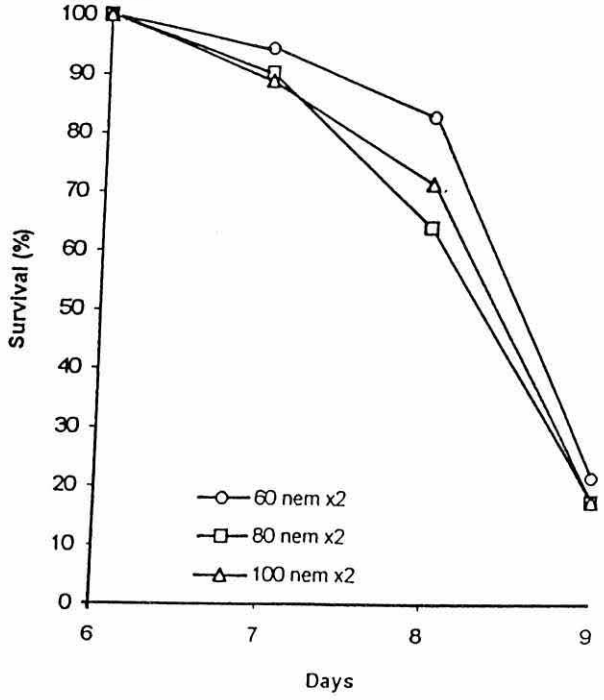
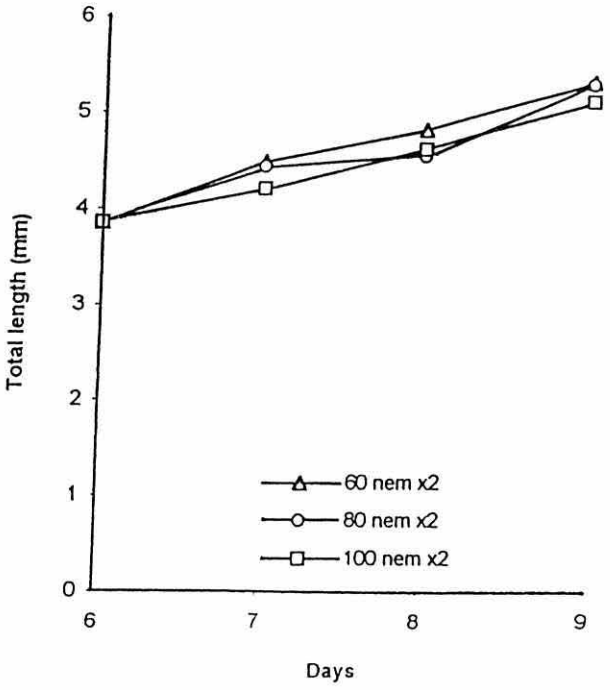


Figure 4b.



Figures 4a and 4b. The survival (%) and total length (mm) of *P. indicus* larvae fed on three densities of nematodes (ml^{-1}) in two distribution times a day from PZ3/M1 to PL stages. All values are means of two replicates.

Experiment 7

Lipid enriched nematodes of various sizes fed to *P. indicus* larvae to PL stages.

PZ1-PZ3/M1

The results shown in Table 12c demonstrated that there were significant differences at the 5% level in mortality rates for larvae fed different nematode diets. No significant differences were observed in survival at the M1 stage between larvae fed either small, large or mixed sized nematode feeds (Table 13a). Larvae fed mixed size nematodes showed significantly lower mortality rate (4.12% day^{-1}) compared to all other treatments. No signficant difference was observed in the mortality rate of larvae fed either large or small nematodes (Table 12c).

M1-PL1

The mortality rate of larvae fed nematodes was not significantly different at the 5% level from each other during mysis and early PL stages (Table 13b). The percentage survival of postlarvae fed mixed (53%), large (44.33%) and small nematodes was significantly different at the 5% level from each other. A lower survival was shown (29.67%) on larvae fed S/L nematodes and was significantly different from all the other treatments. Fig 5b demonstrates survival of the larvae during mysis and early PL stages. Survival data is summarised in tables 13a and 16a .

Growth

PZ1-PZ3/ML1

The growth rates of larvae fed on nematode diets were not significantly different at the 5% level during protozoeal stages. M1 larvae indicated when comparing larval total lengths that mixed size and large nematodes promoted significantly better growth at the 5% level larval total lengths than other nematode feeds at this stage (Table 14b). Small nematodes promoted significantly smaller larval size (4.12 - 4.14) at M1 stage than large nematodes at the 5% level.

M1-PL1

Figure 6b shows growth of the larvae during mysis and early PL stages. From Table 15b there was no significant difference at the 5% level between larval growth rates which ranged between 0.376 and 0.439 mm day^{-1} on different nematode feeds during these stages. The smallest size at metamorphosis was shown by larvae fed small nematodes and was significant at the 5% level . All larvae on the feeding regimes developed into M1 stage on day 6 and started to metamorphose into PL1 stage on day 8-9.

Table 12a. Survival, total length and duration of larval development of *P .indicus* larvae at M1 and PL1 stages on various diets. Values with different superscripts are significantly different (P<0.05). Values are means +/- s.d. (n=3).

	Survival (%)		Total length (mm)		Duration (days)	
Diets	M1	PL1	M1	PL1	toM1	toPL1
Mixed	59.50^{a}+/-3.50	53.00^{a}+/-6.38	4.25^{a}+/-0.12	5.51^{a}+/-0.21	6	8-9
Large	49.33^{ab}+/-5.62	44.33^{ab}+/-3.62	4.20^{a}+/-0.16	5.41^{ab}+/-0.24	6	8-9
Small	50.17^{ab}+/-3.04	47.83^{a}+/-6.64	4.14^{b}+/-0.13	5.27^{b}+/-0.27	6	8-9
S/L	38.33^{b}+/-8.28	29.67^{b}+/-7.00	4.12^{b}+/-0.13	5.35^{ab}+/-0.23	6	8-9

Table 12b. Two-way ANOVA with days as a covariate on survival (%) of *P. indicus* on various feeding regimes from PZ1 (day 2) to PZ3/M1 (day 6) stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	3	1705.37	247.93	82.64	1.83	0.158	NS
Days	1	5908.17	5908.17	5908.17	131.15	0.000	
Repl (Deits)	8	283.52	329.29	41.16	0.91	0.516	
Diets* Days	3	381.45	381.45	127.15	2.82	0.052	
Repl *Days (Diets)	8	209.87	209.87	26.23	0.58	0.786	
Error	36	1621.81	1621.81	45.05			
Total	59	10110.19					

Table 12c. Comparison of mortality rates (% day^{-1}) from PZ1 to PZ3/M1.

Term	Mortality rate (% day^{-1})	Coeff	St dev	T-value	P	Significant
Constant		82.250	2.032	40.47	0.000	
Days		-7.0167	0.6127	-11.45	0.000	
Days*Diets						
Mixed	4.117	2.900	1.061	2.73	0.010	**
Large	6.983	0.033	1.061	0.03	0.975	Ns
Small	8.467	-1.450	1.061	-1.37	0.180	Ns
S/L	8.500	-1.483	1.061	-1.40	0.171	Ns

Table 13a. Two-way ANOVA with days as a covariate on survival (%) of *P. indicus* on various feeding regimes PZ3/M1 (day 6) to PL (day 9) stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	3	3124.27	68.35	22.78	0.64	0.596	
Days	1	182.00	182.00	182.00	5.12	0.033	
Repl (Deits)	8	136.92	108.63	13.58	0.38	0.920	
Diets* Days	3	39.39	39.39	13.13	0.37	0.776	
Repl *Days (Diets)	8	134.78	134.78	16.85	0.47	0.862	
Error	24	852.45	852.45	35.52			NS
Total	47	4469.81					

Table 13b. Comparison of mortality rate (% day -1) from PZ3/M1 to PL stages.

Term	Mortality rate (% day^{-1})	Coeff	St dev	T-value	P	Significant
Constant		57.008	5.075	11.23	0.000	
Days		-1.7417	0.7694	-2.26	0.033	
Days*Diets						
Mixed	2.084	-0.342	1.333	-0.26	0.800	**
Large	1.534	0.208	1.333	0.16	0.877	Ns
Small	0.567	1.175	1.333	0.88	0.387	Ns
S/L	2.784	-1.042	1.333	-0.78	0.442	Ns

Table 14a. Two-Way ANOVA with days as covariate on total length (mm) of *P. indicus* on various feeding regimes from PZ1 (day2) to PZ3/M1 (day 6) stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	3	0.696	0.073	0.024	0.46	0.709	
Days	1	594.687	593.676	593.676	1.1E+0.4	0.000	
Repl (Deits)	8	0.628	0.051	0.006	0.12	0.998	
Diets* Days	3	0.075	0.073	0.024	0.47	0.705	
Repl *Days (Diets)	8	0.159	0.159	0.020	0.38	0.932	
Error	637	33.293	33.293	0.052			NS
Total	660	629.538					

Table 14b. Comparison of the growth rates (mm day^{-1}) from PZ1 to PZ3/M1.

Term	Growth rate (mm day^{-1})	Coeff	St dev	T-value	P	Significant
Constant		0.81604	0.02116	38.57	0.000	
Days		0.669915	0.006286	106.58	0.000	
Days*Diets						
Mixed	0.680	0.00968	0.01096	0.88	0.377	**
Large	0.699	-0.00057	0.01081	-0.05	0.958	Ns
Small	0.672	0.00211	0.01087	-1.03	0.846	Ns
S/L	0.659	-0.01122	0.01092	0.19	0.305	Ns

Table 15a. Two-way ANOVA with days as covariate for total length (mm) of *P. indicus* on various feeding regimes from PZ3/M1 (day 6) to PL (day 9) stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	3	1.8593	0.1857	0.0619	0.64	0.588	
Days	1	122.578	121.853	121.853	1265.62	0.000	
Repl (Deits)	8	1.5205	0.3600	0.0450	0.47	0.879	
Diets* Days	3	0.4108	0.4321	0.1440	1.50	0.215	
Repl *Days (Diets)	8	0.4176	0.4176	0.0522	0.54	0.825	
Error	578	55.6492	55.6492	0.0963			Ns
Total	601	182.436					

Table 15b. Comparison of growth rates (mm day^{-1}) from PZ3/M1 to PL stages

Term	Growth rate (mm day^{-1})	Coeff	St dev	T-value	P	Significant
Constant		2.216780	0.07551	29.37	0/00	
Days		0.40448	0.01137	35.58	0.00	
Days*Diets						
Mixed	0.439	0.03468	0.01936	1.79	0.074	Ns
Large	0.411	0.00713	0.01990	0.36	0.720	Ns
Small	0.376	-0.01392	0.02018	-0.69	0.490	Ns
S/L	0.390	-0.02789	0.01932	-1.44	0.149	Ns

Table 16a. Survival (%) data for figures 5a and 5b . Each value is a mean +/- s.d. n=3.

Days	Mixed	Large	Small	S/L
1	100.00	100.00	100.00	100.00
2	76.50+/-4.82	79.33+/-6.25	89.00+/-2.74	69.67+/-5.88
3	69.00+/-3.50	63.33+/-8.33	64.34+/-1.05	64.17+/-7.51
4	64.67+/-5.01	61.83+/-7.78	62.00+/-6.24	48.33+/-8.75
5	61.83+/-4.37	53.50+/-6.08	57.33+/-3.06	41.83+/-8.52
6	59.50+/-3.50	49.33+/-5.62	50.17+/-3.04	38.33+/-8.28
7	54.83+/-7.29	45.67+/-5.11	48.50+/-6.50	32.83+/-6.71
8	53.50+/-6.25	45.33+/-4.65	47.17+/-3.25	31.00+/-6.38
9	53.00+/-6.38	44.33+/-3.62	47.83+/-6.64	29.67+/-7.00

Table 16b. Growth data (mm) for figures 6a and 6b . Each value is a mean +/- s.d. n=3.

Days	Mixed	Large	Small	S/L
1	1.18+/-0.06	1.18+/-0.06	1.18+/-0.06	1.18+/-0.06
2	1.35+/-0.01	1.37+/-0.01	1.35+/-0.01	1.36+/-0.01
3	2.27+/-0.02	2.26+/-0.03	2.29+/-0.01	2.23+/-0.01
4	2.91+/-0.09	2.88+/-0.10	3.09+/-0.10	3.01+/-0.14
5	3.33+/-0.03	3.38+/-0.07	3.41+/-0.05	3.35+/-0.03
6	4.18+/-0.08	4.17+/-0.09	4.14+/-0.08	4.10+/-0.01
7	4.69+/-0.09	4.80+/-0.14	4.58+/-0.11	4.69+/-0.12
8	5.27+/-0.07	5.09+/-0.17	5.10+/-0.05	5.11+/-0.05
9	5.51+/-0.04	5.41+/-0.01	5.27+/-0.11	5.35+/-0.03

Figure 5a.

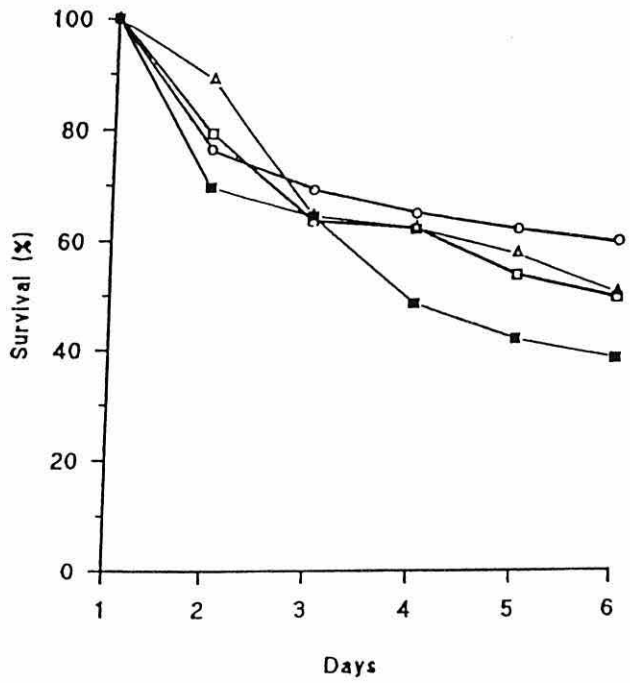
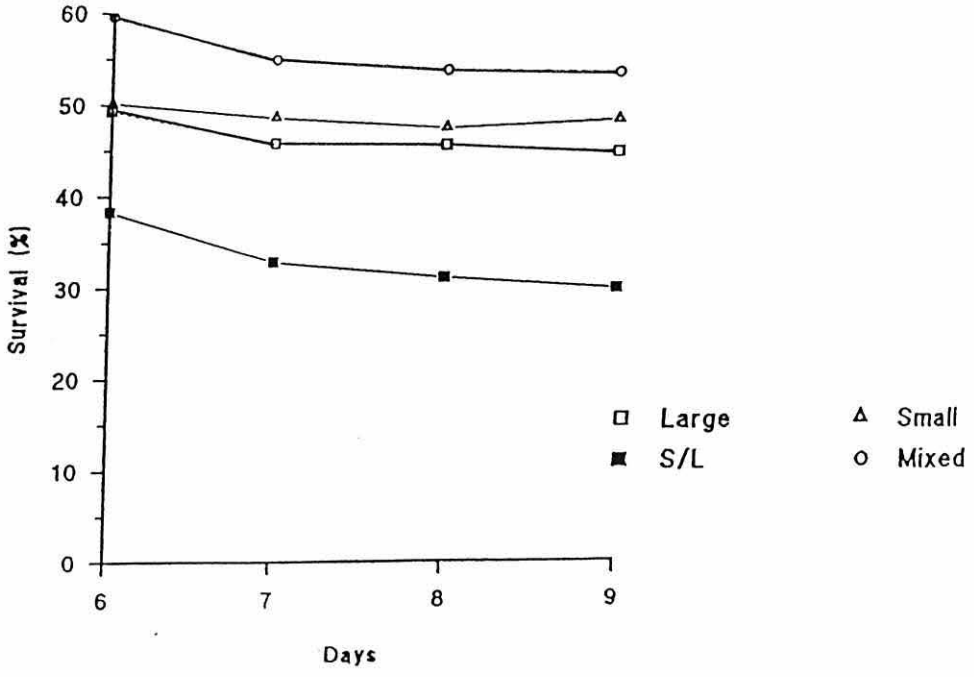


Figure 5b.



Figures 5a and 5b. The survival (%) of *P. indicus* larvae fed on various nematode feeding regimes from PZ1 to PL1 stages. Large, small and mixed size nematodes were fed to the larvae throughout larval stages. For the S/L treatment, the larvae were fed on small nematodes between PZ1 and PZ3/M1 stages and then large size nematodes between M1 and PL1 stages. All values are means from three replicates.

Figure 6a

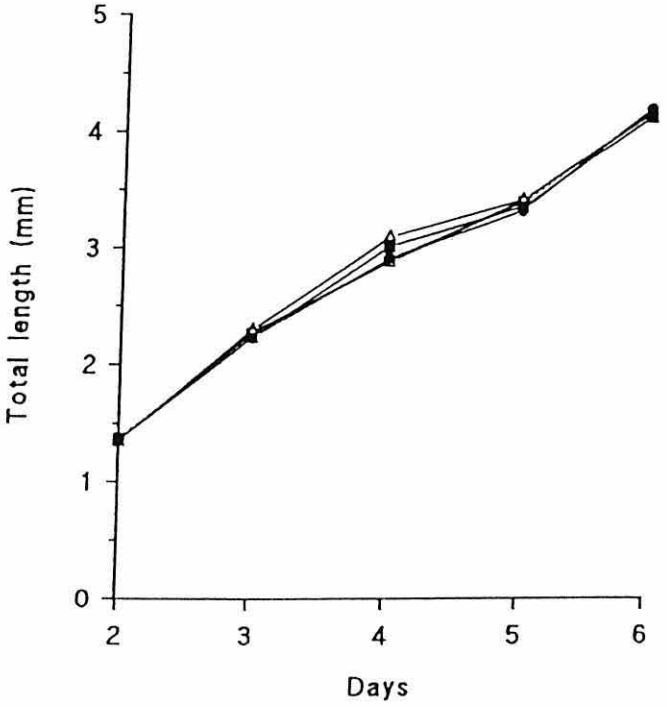
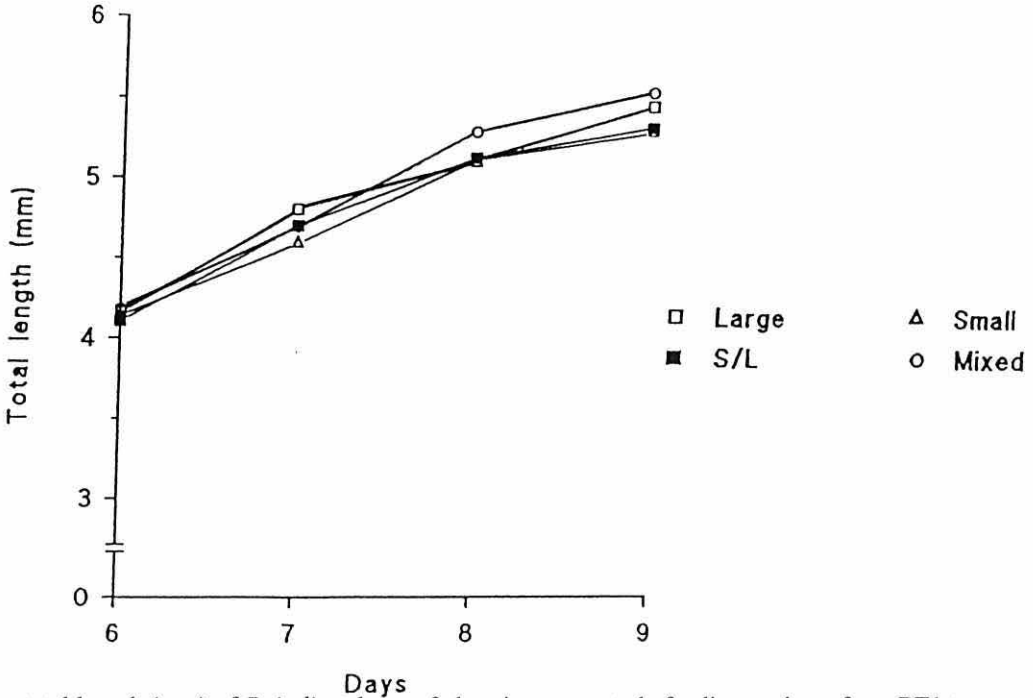


Figure 6b



Figures 6a and 6b. The total length (mm) of *P. indicus* larvae fed various nematode feeding regimes from PZ1 to PL1 stages. Large, small and mixed size nematodes were fed to the larve throughout larval stages. For the S/L treatment, larvae were fed on small nematodes between PZ1 and PZ3/M1 stages and then large size nematodes between M1 and PL1 stages. All values are means from three replicates containing measurements from 10-14 larvae.

DISCUSSION

The objective of this study was to eliminate the use of microalgae and *Artemia* during larval culture of *P. indicus*. Kumlu (1995) has demonstrated that penaeid species can be successfully reared on mixed algae (*T. chuii/S. costatum*) during herbivorial stage and weaned onto *Artemia* nauplii during mysis and early PL stages with or without the presence of microalgae. It has been demonstrated that *Artemia* nauplii can be successfully replaced by the nematode *P. redivivus* in rearing *P. indicus* larvae during mysis and early PL stages.

Equal growth, development and survival was demonstrated by PZ2/PZ3 *P. indicus* larvae fed on algae/ nematode until M2/M3 to that of the algae/*Artemia* and control treatment (Figs 1a and 1b). However larvae at the PL1 stage appeared paler but as healthy as those fed on *Artemia*.

Experiment 1 established that *P. redivivus* was suitable food for *P. indicus* larvae for mysis stages but larvae at the PL stage appeared considerably paler in colouration than the *Artemia* fed larvae. Larvae were observed feeding on the nematodes as faecal strands were present.

It was demonstrated that high mortalities were observed when larvae (PZ1) were fed solely on the nematode regimes (*P redivivus* and *C. elegans*) in experiment 2. It was observed that these nematode cultures were introducing medium into the culture vessels and this directly attributed to the failure of cultures in experiment 2, 3 and 4. This resulted in the deterioration of the culture water and immediate larval fouling. It was therefore established that for the use of nematodes in penaeid larval culture, they must be free of the culture medium.

Wilkenfeld et al (1984) determined the feeding level of nematodes which would give equal protein to that of *Artemia* ml^{-1}, by using 70 nematodes ml^{-1} of *P. redivivus* to rear *P. aztecus*, *P. setiferus* and *P. vannamei* during larval development. Biedenbach et al (1989)studied feeding levels of nematodes for *P. vannamei* at different larval stages with algae in the larval culture system. The nematode concentrations tested in experiment 4 (15, 30, 45 and 60 nematodes ml^{-1} day^{-1}) with *P. indicus* larvae between PZ2/PZ3 to PL1 stages showed that the lowest concentration of nematodes produced the lowest growth rates (0.463 mm day^{-1}) compared to

those fed higher concentrations. There was no advantage feeding 30, 45 and 60 nematodes ml^{-1} as growth rate was not significantly different at the 5% level. Biedenbach et al., (1989) also found non-significant differences between dry weight of *P. vannamei* when they fed the larvae on various nematode concentrations ml^{-1} and larval stages.

The results presented in experiment 5 indicate that 30 nematodes ml^{-1} when given twice daily (15 nematodes ml^{-1}) promoted a higher survival (71%) than 50 nematodes ml^{-1} (fed twice daily). Higher nematode concentrations on either 1 or 2 meals a day caused pollution of the culture water and clumping of the nematodes with each other and onto the larvae. On the last day of the experiment all nematode feeding regimes caused unexpected high mortalities, which may have been due to the fungal or bacterial contamination occasionally observed during the culture of the nematodes in flasks and fermenters. However before the contamination was observed larvae fed 60 nematodes ml^{-1} on 2 meals per day promoted consistently better larval survival.

Biedenbach et al (1989) also supported the present findings that concentrations of nematodes from 25 to 300 nematodes ml^{-1} produced similar dry weights at PL stage of *P. vannamei.* Therefore it is proposed that 30 nematodes ml^{-1} produced similar protozoeal stages (PZ1-PZ3), 45 nematodes ml^{-1} during M1-M2 and 60-70 nematodes ml^{-1} between M3-PL1, in two meals a day are suitable to rear *P. indicus* larvae with superior survival, although growth and development may be still inferior to the standard algae/ *Artemia* diet.

Live nematodes have several advantages over algae and *Artemia*, for example unlike *Artemia* nematodes do not consume algae, never grow too large to be consumed by the larvae and can survive in sea water upto 72h (Fontaine et al., 1992) cited in Biedenbach et al., 1989). Nematodes can be cultured on several types of media (Chapter 1) and particularly in liquid medium which allows them to be produced in large quantities in fermenter vessels for commercial purposes. They have very high reproduction rates and a nutritive value that can be manipulated to be comparable to that of *Artemia* (Chapter 2). Kahan et al., (1980), Leger and Soorgeloos, (1992) mention a comparable nutritive value by the introduction of lipids and pigments (Chapter 3). The nutritive value of nematodes may be kept relatively consistent in comparison to microalgae and *Artemia* which is known to be inconsistent in nutritional quality

(Watanabe et al, 1983, and Leger et al., 1986) and may cause a considerable risk to productivity in commercial penaeid larviculture. However nematodes require a continuous aeration system to maintain optimum predator prey interaction and therefore an aeration system may be required in larger culture vessels.

It can be concluded that the present results show that *P. redivivus* is a suitable alternative diet for *P. indicus* larvae both as an algae and *Artemia* replacement in small culture vessels and should seriously be considered for commercial penaeid hatcheries.

Wilkenfeld et al., (1984) suggested nematodes could partially or completely replace *Artemia* nauplii in the diets of some penaeid species without reducing growth, survival or metamorphic rate. Nematodes have been used on a limited basis in some commercial larviculture facilities, however nematode feeding levels required by the larvae for good growth have not been well defined. Previous studies investigating nematodes as food for penaeid larvae have had mixed results, possibly due to the low levels of nematodes used e.g. Fontaine et al., (1982) obtained variable survival of *P. stylirostris* using frozen nematodes at a static rate of $30ml^{-1}$. Wilkenfeld et al (1984) obtained better survival with larval penaeids when they were fed live nematodes at a rate of $70ml^{-1}$, disproving Fontaine's previously reported explanation that penaeid larvae could not capture and consume live nematodes. Wilkenfeld et al., (1984) recognised the importance of growth as an indicator of the performance of the diet and suggested that penaeid species such as *P. vannamei* are more sensitive to an animal food source in feeding regimes containing an inferior algal source. *Artemia* consistently produced greater growth to post larvae than diets containing static levels of nematodes. The work by Wilkenfeld et al., (1984) suggests that in the presence of sufficient quantities of nematodes with algae, final biomasses of *P. vannamai* postlarvae can equal those obtained on a diet of *Artemia* with algae. Biedenbach et al., (1989) estimated the minimum quantities of nematodes to be fed in conjunction with algae. These authors concluded that the production of commercially important penaeid seed using nematode/algal feeding regime could equal that obtained under the generally accepted normal method of feeding *Artemia* and algae. However solid media culture techniques for nematodes used in these experiments precluded development of large scale trials.

CHAPTER SIX

SHRIMP LARVAL GROWTH TRIALS ON LIPID ENRICHED AND PIGMENTED NEMATODES.

The following experimental work was undertaken at Bangor University under the supervision of Dr. David Fletcher and part of this work has been published as follows:

Fletcher, D.J., Kumlu, M. and Fisher, C.M. 1995. Growth and survival of *Penaeus indicus* larvae fed on the nematode *Panagrellus redivivus* enriched with astaxanthin and various marine lipids. *Book of Abstracts of the World Aquaculture '95', San Diego, February 1-4, 1995.* World Aquaculture Society, Baton Rouge, Louisiana, U.S.A. pp.117

Kumlu, M., Fletcher, D.J., and Fisher, C.M. 1997. Larval pigmentation, survival and growth of *Penaeus indicus* on the nematode *Panagrellus redivivus* enriched with astaxanthin and various lipids. To be published in *Aquaculture Nutrition.*

6. SHRIMP LARVAL GROWTH TRIALS ON LIPID ENRICHED AND PIGMENTED NEMATODES

INTRODUCTION

The results obtained in chapter 5 have demonstrated that the nematode *P. redivivus* may be a suitable alternative to algae and *Artemia* in the larval rearing diet of *P. indicus*.

Present work demonstrates that nematodes cultured on liquid media capable of scale up to 2000L can sustain survival and growth of penaeid larvae similar to that of other live feeds. However experimental work undertaken using nematodes as a live feed replacement for caridean larvae, *P. elegans* and *M. rosenbergii* showed that they were unable to grow and survive on the nematodes (Kumlu, 1995). A preliminary experiment with the native prawn *P. elegans* showed that larvae of this species were not capable of catching and ingesting nematodes. Experiments with the fresh water prawn *M. rosenbergii* demonstrated that the nematodes are not suitable as a food for caridean larvae.

Higher nematode densities were used (75 - 150 nematodes ml^{-1}) in the first experiment but larval guts remained empty. A larger nematode species *S. feltiae* fed at higher densities (150-225 ml^{-1}) did not improve survival and also caused a deterioration in water quality. At low feeding rates with *P. redivivus* (10 - 30 ml^{-1}) larvae of *M. rosenbergii* displayed poor survival and growth similar to that of the starved control. These results suggest that the failure of carnivorous caridean larvae to capture insufficient nematodes may be due to cylindroconical body shape, sinusoidal movement and size of the nematodes. It was not thought to be due to toxic or noxious substances as larvae did not die earlier than starved ones and also some larvae were observed with nematodes in their guts. Inappropriate nutritional content of the nematodes seems unlikely since nematodes have been proved to be perfectly adequate as food for various penaeid sps. such as *P. aztecus*, *P. setferus*, *P. vannamei* (Wilkenfeld et al., 1984; Biedenbach, 1989) and *P. indicus* (present study).

However although results in chapter 5 showed that nematodes promote high larval survival in

P. indicus larvae, they give relatively inferior growth and development compared to an algae/*Artemia* feeding regime. The present study investigates the suitability of the nematode *P. redivivus* by using different methods to enhance their nutritional value for larval culture of *P. indicus*. Several live algal co-feeds with nematodes were fed to the larvae in an attempt to elevate larval digestive enzymes (Jones et al., 1993; Le Vay et al., 1993;) and hence to improve the digestion of the prey (Hofsten et al., 1983). Nematodes which had been enriched with a lipid source (capelin oil) to improve their essential fatty acid (EFA) profiles, particularly eicosapentaenoic acid (20:5ω-3) and docosahexaenoic acid (22:6ω-3) were tested to examine effects on growth, survival and development of the larvae. Finally nematodes pigmented with astaxanthin were fed to *P. indicus* larvae to improve the pigmentation of postlarvae equivalent to those fed on algae and *Artemia*. The effects of the pigment on larval survival and growth during larval development (PZ1-PL1) of *P. indicus* were also investigated.

MATERIALS AND METHODS

Rearing procedures for *Penaeus indicus* larvae.

For each experiment larvae were obtained from individual females held in 50 l spawning tanks at 28^{0}C and 33pp salinity. 50% of the sea water was changed after the spawning and irradiated with UV light to reduce bacterial contamination and cartridge filtered through 0.2µm to eliminate food sources from the larvae. Protozoea (PZ1) were given a 15 min bath in an antibiotic (furazolidone) at 0.2ppm before larvae were stocked in experimental 2 l round bottomed flasks incubated at 28^{0}C +/- 0.5^{0}C(thermostatically controlled) and containing a larval density of 100 larvae l^{-1}. Optimum predator prey interaction and O_2 circulation was maintained without damage to the prawn larvae by contiuous aeration supplied via silicone rubber tubing with glass tubes at the tip. Complete water exchanges were performed every 2 days when the larvae were also counted and a sample 20 were measured in length and staged. 50% water changes were made on alternate days. Live food counts were performed on individual flasks and appropriate amounts of the feed were added to maintain specific feeding regimes detailed below. Samples of the larvae were measured at the commencement of each trial. *Artemia* cysts supplied by (INVE

AQUACULTURE), Belgium) were continously hatched at 26 -28^{0}C at 32ppt salinity during the experiment. The nauplii were separated from the shells of the cysts and counted to estimate their density. Salinity for each experiment was maintained at 25ppt (optimal to rear *P. indicus* larvae) for all experiments by mixing filtered (0.2µm) and UV sea water with distilled water. All nematodes were cultured by myself. Complete water changes were carried out everyday when 10-13 larvae were sampled randomly to determine growth of the larvae as increase in total length (mm) and larval development. Every other day, a new nematode culture was introduced to the larvae after they were rinsed in filtered and UV treated sea water and counted in a rafter cell counter.

Nematode cultures

Live nematodes were soaked in small cubic sponges (see chapter 4 : 10 million in 2g sponge in 10ml of water) and kept at 4^{0}C in an ordinary refrigerator. They were released in distilled water, concentrated and counted (three times) in a 1ml counting chamber before they were fed to the larvae. The size range of the nematodes was generally between 150µ and 900µ. Nematodes were produced in a liquid medium monoxenically with attempts to manipulate their nutritional profile to supply a more adequate food for penaeid larvae. Culture conditions of the nematodes were;

Bacteria : *Escherichia coli* (OP50)

Medium (w/w): 10% kidney, 1% yeast extract and 3.5% corn oil

Flasks : 250 ml baffled flasks with 50ml of medium,

Culture temperature : 22^{0}C.

Shaking incubator : 170 rpm.

An inoculum of 2000 nematodes ml^{-1} was added to the flasks which had been previously (24h) inoculated with *Escherichia coli.* The flasks were incubated at 22^{0}C for 10 -22 days in an orbital incubator, at approximately 150, 000 nematodes ml^{-1}. The nematodes were extracted from their medium by sedimentation and decantation in fresh water. They were packed in 15 x 18 cm high density polyethylene bags containing 2g of foam blocks and 10-20 ml of water. When large numbers of nematodes were required, they were produced in mass quantities in fermenters (10

litre or larger). These vessels were inoculated with 2000 nematodes ml^{-1} and harvested when the population reached a maximum of 174,000 nematodes ml^{-1} in 18-25 days. Generally, nematodes were regularly obtained from AGC and used in 1-3 days of receipt from the company during the experiments.

P. redivivus is a terrestrial nematode and it was shown to lack some essential fatty acids particularly 20:5ω3 and 22:6ω3 (HUFA) that penaeids require in their diets (Jones et al., 1979). For some of the following experiments 50% of the corn oil was replaced capelin oil to enrich the fatty acid profile of the nematodes (as described in chapter 2). Carophyll pink (Hoffman La Roche Ltd., Switzerland), which contains 8% astaxanthin, was added to the cultures in an attempt to improve the pale colour of the larvae normally obtained with the non pigmented nematodes (as described in chapter 3). The possible physiological effects that the pigment might have of the larvae were also observed. The pigment in the form of gelatine/starch microcapsules was dissolved in the corn oil fraction of the medium and autoclaved before adding to the general culture medium. The experimental work undertaken in chapter 3 shows that 1.5% (w/w) Carophyll Pink, which provides $1.43\mu g^{-1}$ astaxanthin g^{-1} dry weight of the nematodes, was the maximum level that can be used in the medium to pigment the nematodes.

Lipid and fatty acid analysis was undertaken as described in the material and methods of chapter 2. Protein analysis was undertaken by Dr. Steven Mudge at Bangor at the University of North Wales using the standard Micro KJ ELDAHL (Foster and Gabbott, 1971).

Experiment 1.

The survival and growth of *P. indicus* larvae fed on a variety of enriched nematode/algal co-feeds.

This experiment investigates the survival and growth rate of *P. indicus* larvae fed on a variety of enriched nematode/algal co-feeds. *P. indicus* larvae were fed the following *nematode/algal feeds:-

a) Lipid-enriched nematodes (EN)

b) Non-enriched nematodes (NEN)

c) Lipid-enriched pigmented nematodes (PE)

d) Non-enriched pigmented nematodes (PNE)

e) Enriched nematodes plus mixed algal (*T.chuii/S.costatum*, 1:1) co-feed 30 cells/ul for 24 hours (EN / Alg)

f) Control treatment (25 cells μl^{-1} T. chuii, 45 cells μl^{-1} *S. costatum* and plus five *Artemia* ml^{-1} during mysis and postlarval stages.

The survival data did not fit in the GLM, logarithmic transformation was carried out before the data was analysed by two-way ANOVA with days as a covariate from PZ1 to PL stages. Larval growth was also analysed by the same method but separately between PZ1 and PZ3/M1, and between M1 and PL stages in order to compare the performance of larvae on the nematode diets against the larvae fed algae during protozoeal stages and those cultured on *Artemia* nauplii during later stages. Final larval surivival (at PL) and growth (total lenghts) were also compared using one-way ANOVA and appropriate pairwise comparison tests (Schéffe's test and Tukey's test) following Bartlett's homogeneity test for variances.

Experiment 2

Determination of the effects of nematode lipid enrichment using capelin oil and pigmentation on the growth and survival of *P. indicus* larvae.

Results obtained from experiment 1showed that larval enhancement was demonstrated, however reliable data was not obtained to show any positive effects of the lipid and/or pigment enhancement. Difficulty in the supply of nematodes and the extraction of nematodes from the culture medium were to blame. Nematodes when cultured in a medium containing astaxanthin were very difficult to extract and therefore required extensive cleaning procedures. Therefore experiment 1 was repeated to determine the effects of lipid enrichment (capelin oil) and pigmentation on survival and growth of *P. indicus* larvae. Three different algal co-feeds along

with the nematodes were also tested on larval growth, survival and to determine the best algal density and algal co-feeding duration required to obtain comparable growth, development and survival to those obtained from the control diet. The following feeding regimes were used to rear PZ1 larvae until PL stages.

a) Non-enriched nematodes (NEN)
b) Lipid-enriched (EN)
c) Lipid-enriched/pigmented (PEN)
d) Pigmented and non-lipid-enriched nematodes (PNE)
e) Lipid-enriched plus 30 cells μl^{-1} mixed algae (*T. chuii/S. costatum*, 1:1) for 24 h (EN/Alg1)
f) Lipid-enriched nematodes plus 30 cells μl^{-1} mixed algae (*T. chuii/S. costatum*, 1:1) for 48h (EN/Alg2)
g) Lipid-enriched nematodes plus 15 cells μl^{-1} mixed algae (*T. chuii/S. costatum*, 1:2) for 48 h (EN/ Alg 3)
h) Control treatment (25 cells μl^{-1} *T. chuii*, 45 cells μl^{-1} *S.costatum* and plus five *Artemia* ml^{-1} during mysis and postlarval stages.

Nematode concentrations offered to the larvae during larval development were 30 nematodes ml^{-1} (PZ1-PZ3/M1), 45 nematodes ml^{-1} (M1-M2), 60 nematodes ml^{-1} (M3-PL). PZ1 stage larvae were fed on the EN nematodes, EN/Alg3, and mixed algae (60 cells μl^{-1}) for 48 h. Protein lipid and fatty acid profiles of the nematodes used in the present study were supplied by myself (Table 13). Nematode cultures were used within 1-2 days from receipt from AGC.

Survival data was compared using two-way ANOVA with days as a covariate between day 2 (PZ1/PZ2) and day 10 (PL1-P4) using GLM. Growth data, however, were compared separately from PZ1-PZ3/M1 (day1 to day 4) and from M1 to PL stages (day 5 to day 10) using the same method above. Furthermore, final larval survival and growth (at PL stages) were also compared using one-way ANOVA followed by appropriate pairwise comparison tests (Schéffe's and Tukey's tests).

Experiment 3

The effects of nematodes (*P. redivivus*) enriched with different lipid sources on larval growth and survival of *P. indicus*

The effects of *P. redivivus* enriched with different lipid sources on larval growth and survival of *P. indicus* were compared in this experiment. Nematode cultures were used within 1-2 days of receipt from AGC. Five different diets were tested from PZ1 to PL1 stages as follows;

a) Marilla lipid-enriched nematodes (MAR)
b) Cod liver lipid enriched nematodes (CLO)
c) Capelin lipid-enriched nematodes (EN)
d) Non-enriched nematodes (NEN)
e) Control diet: 25 cells μl^{-1} *T.chuii* and 35 cells μl^{-1} *S.costatum* and five nauplii ml^{-1} from M1 to PL1. The algal diet was completely ceased at M1/2 stage.

The nematodes were fed to the larvae at a concentration of 30 ml^{-1} at PZ1-PZ2/3, 45 ml^{-1} PZ3/M1-M2, and 60 ml^{-1} M3-PL1. The protein, lipid and fatty acid profile of the nematodes used as food to rear *P. indicus* larvae in this experiment were analysed. Capelin oil, cod liver oil, and marilla oil were used to replace 50% of the corn oil in culturing the nematodes to elevate HUFA level to that required by penaeid larvae.

Growth and survival data was analysed using two-way ANOVA with days as a covariate (GLM) to compare growth rate and survival rate of larvae between PZ1 to PZ3/M1 stages (day 2-day 6), and M1-PL stages (day 6-day10). Comparison of larval growth and survival at M1 and PL1 stages were performed using one-way ANOVA followed by Schéffe's and Tukey's pairwise comparison tests after the data was checked for homogeneity of variances using Bartlett's test.

Experiment 4

The effects of nematodes (*P. redivivus*) enriched with either pigment or a placebo on *P. indicus* larvae.

In experiment 3, the use of the pigment astaxanthin with regards to larval growth and survival, was inconclusive. Consequently, the experiment was repeated using nematodes enriched either with the pigment or the placebo pigment capsules. So that the effect of the pigment plus gelatine capsule or gelatine capsule alone could be demonstrated. Three diets were tested;

a) Pigmented and lipid-enriched (PEN)

b) Placebo pigmented and lipid-enriched (PLC), and

c) Lipid-enriched (CLO).

d) Control larvae were cultured on the live mixed algae (25 cells μl^{-1} *T. chuii*/45 cells μl^{-1} *S. costatum*) and five *Artemia* ml^{-1} during mysis and PL stages.

All other experimental procedures were identical to those in Experiment 3. Growth and survival data was collected from three replicates for each treatment. Protein, lipid and fatty acid profile of the nematodes fed to the larvae were analysed.

Survival rates and growth rates were compared using two-way ANOVA with days as a covariate (GLM) from PZ1 to PZ3./M1 and from M1 to PL stages separately. To fit in the model (GLM) survival rates during protozoeal stages were compared between day 3 and day 6. Following Bartlett's homogeneity test, growth and survival data at M1 and PL1 stages were analysed with one-way ANOVA in minitab. Further pair wise comparison tests were conducted using Schéffe's test for growth and Tukey's test for survival.

RESULTS

Experiment 1

The survival and growth of *P. indicus* larvae fed on a variety of enriched nematode/algal co-feeds.

Results presented in Table 5 show the fastest larval development was achieved with the control feed followed by the EN/Algae both during the protozoeal and later stages. On day 4: 1) all larvae fed on algae were at M1 stage whereas only 73% of these fed EN/algae were at M1 and the remaining 37% at PZ3 stage; 2) 62% of larvae reared on PEN nematodes were at M1 compared to 13% of those reared on NEN nematodes. At the termination of the experiment , 35% of the control larvae were at PL3/PL4 stages. On day 10 EN/algae and PEN nematode diets supported fast development (PL1/PL2) compared to other diets (M3/PL1).

Survival

The results obtained for the survival data did not fit in the GLM model and therefore logarithmic transformations were carried out before survival rate of larvae reared on three different feeding regimes were compared between day 4 and day 10.

The results of this experiment demonstrated that a complete replacement of live algae and *Artemia* in the culture of *P. indicus* throughout all larval stages to metamorphosis can be achieved successfully using the nematode *P. redivivus* alone. The results shown in Table 2a reveal significant differences of the treatments for larval survival at the 1% level. Table 2a and 2b demonstrate the lowest log mortality rate at the 1% level achieved with lipid enriched nematodes combined with 30 cells μl^{-1} algal co-feed (EN/algae) from PZ1 to PL1 stage. In Table 1 no significant differences were observed in the mortality rate of larvae fed on the control diet and those reared on PNE nematodes. Comparisons of the final survival at the PL stage on day 10 demonstrates that EN/Algae supported significantly higher larval survival (72%) at the 5% level compared to all other feeding regimes. Figure 1 shows that there were no significant differences at the 5% level observed in larval survival between NEN (57%), PNE (50%), control

(49%) and EN (42%) nematodes at PL stages. PEN nematodes displayed the lowest survival of 26% at metamorphosis.

Figure 1, 2a and 2b demonstrate survival and growth of larvae of *P. indicus* reared on various nematode diets and control diets from PZ1 to PL stages. It was difficult to obtain clean nematode culture of PEN and PNE nematodes compared to NEN nematodes and EN nematodes and this resulted in the fouling of the larvae during their culture resulting in high mortalities.

Growth

PZ1-PZ3/M1

Table 3a shows significant differences at 1% level in the growth rate of the larvae on various feeding regimes. Tables 3b and 1 show the highest larval growth rate at the 1% level (0.72 mm day^{-1}) was achieved with mixed algae with a 3.98 mm in total length at the PZ3/M1 stage. The growth rate of larvae fed 30 cells μl^{-1} of algae (*T. chuii/S.costatum*, 1:1) for only 24h together with lipid enriched nematodes was significantly better (0.62 mm day^{-1}) than the larval growth rate obtained for EN nematodes (0.51 mm day^{-1}). The difference in the colouration between the larvae fed pigmented nematodes and non pigmented nematodes could clearly be seen with the naked eye and under microscopic examination as early as PZ1. However the pigmented nematodes were not observed to improve the larval growth rate (0.44-0.56 mm day^{-1}) compared to non-pigmented nematode fed larvae (0.48-0.50 mm day^{-1}). The poorer results obtained with the PEN nematodes could have been due to the dirty nematode cultures. From PZ1 to PZ2 nematodes enriched with capelin oil (EN) had relatively high survivals. However at stage PZ3 larvae displayed the abnormal L shaped body which characterises a nutrient defiency rather than an environmental problem, and as a result growth and development was impaired.

M1-PL

Table 1 shows no significant differences at the 5% level between lipid enrichment and/or pigmentation of the nematodes on larval growth. Tables 4a and 4b demonstrates larval growth rate (0.55mm day^{-1}) and final total length (6.90mm) of larvae fed *Artemia* were significantly

better than larvae fed on the other nematode diets at the 1% level. Tables 6a and 6b shows the growth and survival data. Final total lengths at PL stages were significantly greater (5.68 mm) at the 5% level for larvae fed on algal co-feeds for a period of 24h at PZ1. This was signficantly lower at the 5% level (0.30 mm day^{-1}) compared to those larvae fed on other nematode diets during mysis stages.

Table 1. Growth rate (mm day^{-1}), log mortality rate (% day^{-1}), final survival (%) and total length (mm) of *P. indicus* larvae reared on various nematode feeding regimes and a control diet from PZ1 to PL stages. Treatments with the same superscripts are not significantly different (P>0.05). Value for survival and growth are means +/- s. d. (N=3). NEN = non enriched nematodes, EN = lipid enriched nematodes, PNE = pigmented non enriched, PEN = pigmented enriched nematodes, EN/algae = lipid enriched nematodes fed with 30 cells μl^{-1} of algae (*T. chuii/S.costatum*, 1:1)

Diets	log mortality rate (% day^{-1})	Final survival at PL (%)	Growth rate (mm day^{-1})		Final growth at PL (mm)
			PZ1-PZ3/M1	M1-PL	
NEN	0.023^{d}	56.67^{b}+/-2.52	0.484^{d}	0.328^{c}	5.10^{c}+/-0.14
EN	0.047^{a}	42.17^{b}+/-2.25	0.505^{d}	0.331^{c}	5.18^{c}+/-0.14
PNE	0.026^{c}	49.67^{b}+/-1.26	0.441^{d}	0.349^{b}	5.01^{c}+/-0.05
PEN	0.043^{b}	26.00^{c}+/-2.65	0.560^{c}	0.328^{c}	5.29^{c}+/-0.09
EN/algae	0.018^{e}	71.50^{a}+/-3.54	0.623^{b}	0.304^{d}	5.68^{b}+/-0.09
Control	0.031^{c}	49.33^{b}+/-2.25	0.716^{a}	0.548^{a}	6.90^{a}+/-0.13

Table 2a. Two-way ANOVA with days as a covariate on log larval survival of *P. indicus* from PL1 to PL stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	5	1.101554	0.113816	0.022763	12.45	0.000	
Days	1	0.712966	0.712966	0.712966	390.09	0.000	
Repl(Diet)	12	0009512	0.010791	0.000899	0.49	0.911	
Diet *Days	5	0.076817	0.013464	0.015363	8.41	0.000	***
Repl*Days (Diet)	12	0.014364	0.001122	0.001122	0.61	0.821	
Error	54	0.098697	0.001828	0.001828			
Total	89	2.013010					

Table 2b. Comparison of log larval mortality rates (% day^{-1}) of *P. indicus* fed on various feeding regimes from PZ1 to PL stages.

Term	log mortality rate (% day^{-1})	Coeff	Stdev	T-value	P	Significant
Constant		1.95958	0.01057	185.42	0.000	
Days		-0.06294	-0.003187	-19.75	0.000	
Days*Diet						
NEN	0.023	0.016623	0.007125	2.33	0.023	*
EN	0.047	-0.016623	0.007125	-4.41	0.000	***
PNE	0.026	0.010830	0.007125	1.52	0.134	Ns
PEN	0.043	-0.022344	0.007125	-3.14	0.003	**
EN/algae	0.018	0.026081	0.007125	3.66	0.001	***
Control	0.031	0.000232	0.007125	0.03	0.974	Ns

Table 3a. Two-way ANOVA with days as a covariate on larval growth of *P. indicus* from PZ1 to PZ3/M1 stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	5	66.935	0.550	0.110	1.68	0.138	
Days	1	569.820	568.990	568.990	8679.88	0.000	
Repl(Diet)	12	1.348	0.119	0.010	0.15	1.000	
Diet *Days	5	15.758	15.777	3.155	48.13	0.000	***
Repl*Days (Diet)	12	0.454	0.454	0.038	0.58	0.862	
Error	893	58.539	58.539	0.066			
Total	928	712.854					

Table 3b. Comprisons of larval growth rates (mm day^{-1}) of *P. indicus* fed various diets from PZ1 to PZ3/M1.

Term	Growth rate (mm day^{-1})	Coeff	Stdev	T-value	P	Significant
Constant		1.02143	0.01985	51.45	0.000	
Days		0.55467	0.00595	93.17	0.000	
Days*Diet						
NEN	0.484	-0.07098	0.01317	-5.39	0.000	***
EN	0.505	-0.05016	0.01331	-3.77	0.000	***
PNE	0.441	-0.11401	0.01339	-8.51	0.000	***
PEN	0.560	0.00558	0.01335	0.41	0.681	Ns
EN/algae	0.623	0.06828	0.01309	5.22	0.000	***
Control	0.716	0.16129	0.01336	12.07	0.000	***

Table 4a. Two-way ANOVA with days as a covariate on larval growth of *P. indicus* between M1 and PL stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	5	270.933	8.073	1.615	10.84	0.000	
Days	1	441.792	440.302	440.302	2955.37	0.000	
Repl(Diet)	12	5.738	1.132	0.094	0.63	0.815	
Diet *Days	5	23.108	22.794	4.559	30.60	0.000	***
Repl*Days (Diet)	12	2.009	2.009	0.167	1.12	0.336	
Error	1114	165.967	165.967	0.149			
Total	1149	909.548					

Table 4b. Comparison of larval growth rates (mm day^{-1}) of *P. indicus* on various diets (M1-PL stages).

Term	Growth rate (mm day^{-1})	Coeff	Stdev	T-value	P	Significant
Constant		1.89668	0.05145	36.87	0.000	
Days		0.36485	0.006711	54.36	0.000	
Days*Diet						
NEN	0.328	-0.03608	0.01484	-2.43	0.015	*
EN	0.331	-0.03374	0.01499	-2.25	0.025	*
PNE	0.349	-0.01574	0.01489	-1.06	0.291	Ns
PEN	0.328	-0.03691	0.01528	-2.42	0.016	*
EN/algae	0.304	0.18355	0.01501	-4.04	0.000	***
Control	0.548	-0.06108	0.01503	12.22	0.000	***

Table 5. Percentage larval stages at PZ3/M1 and PL stages of *P. indicus* reared on various feeding regimes from PZ1 to PL stages.

Diets	Larval stages (%) at PZ3/M1 (Day 4)	Larval stages (%) at PL (Day 10)
NEN	87.5% PZ3, 12.5% M1	47% M3, 53% PL1
EN	75% PZ3, 25% M1	40% M3, 60% PL1
PNE	97% PZ3, 3% M1	63% M3, 37% PL1
PEN	38% PZ3, 62% M1	18% M3, 72% PL1, 10% PL2
Control	100% M1	28% PL1, 37.5% PL2, 19% PL3, 15.5% PL4
EN/algae	37% PZ3, 73% M1	81% PL1, 19% PL2

Table 6a. Survival data (%) for Figure 1. Each value is a mean +/- s.d. (n=3).

Days	NEN	EN	PNE	PEN	Control	Nem/algae
0	100.00	100.00	100.00	100.00	100.00	100.00
2	86.50+/-3.28	97.17+/-0.58	83.50+/-4.27	60.50+/-3.78	88.83+/-5.69	100.00
4	69.67+/-1.61	73.67+/-4.80	63.88+/-2.02	38.33+/-4.16	66.33+/-5.69	87.17+/-6.81
6	62.16+/-5.13	58.00+/-4.09	56.67+/-2.03	30.00+/-3.04	61.00+/-1.32	77.00+/-4.77
8	57.88+/-3.75	44.67+/-3.79	54.33+/-3.33	27.50+/-3.78	50.67+/-2.26	73.00+/-5.00
10	56.67+/-2.52	42.17+/-2.25	49.67+/-1.26	26.00+/-2.65	49.33+/-2.25	71.50+/-3.54

Table 6b. Growth data (mm) for figures 2a, b. Each value is amean +/- s.d. (n=3).

Days	NEN	EN	PNE	PEN	Control	Nem/algae
0	1.02+/-0.07	1.02+/-0.07	1.02+/-0.07	1.02+/-0.07	1.02+/-0.07	1.02+/-0.07
1	1.38+/-0.01	1.44+/-0.01	1.38+/-0.01	1.41+/-0.01	1.49+/-0.02	1.48+/-0.03
2	2.16+/-0.02	2.21+/-0.02	2.17+/-0.01	2.20+/-0.03	2.48+/-0.08	2.39+/-0.06
3	2.38+/-0.04	2.43+/-0.03	2.33+/-0.06	2.43+/-0.06	3.35+/-0.11	3.22+/-0.14
4	3.13+/-0.02	3.19+/-0.07	3.12+/-0.02	3.25+/-0.07	3.98+/-0.08	3.66+/-0.08
5	3.36+/-0.04	3.45+/-0.01	3.35+/-0.04	3.67+/-0.09	4.25+/-0.14	4.11+/-0.07
6	3.93+/-0.07	3.88+/-0.03	3.65+/-0.09	4.04+/-0.10	4.68+/-0.04	4.49+/-0.12
7	4.18+/-0.16	4.06+/-0.12	3.85+/-0.07	4.18+/-0.14	5.45+/-0.13	4.64+/-0.07
8	4.42+/-0.10	4.45+/-0.06	4.46+/-0.11	4.68+/-0.04	6.04+/-0.10	5.00+/-0.17
9	4.93+/-0.09	4.80+/-0.10	4.65+/-0.17	5.09+/-0.04	6.56+/-0.04	5.41+/-0.18
10	5.10+/-0.14	5.18+/-0.14	5.01+/-0.05	5.29+/-0.09	6.90+/-0.13	5.68+/-0.09

Figure 1

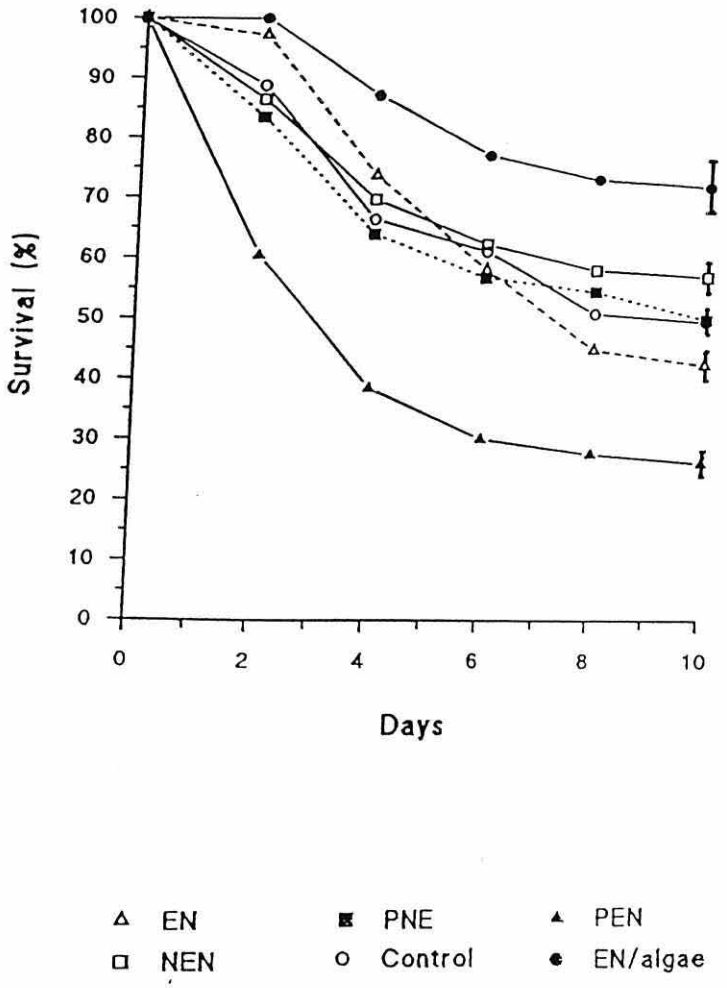
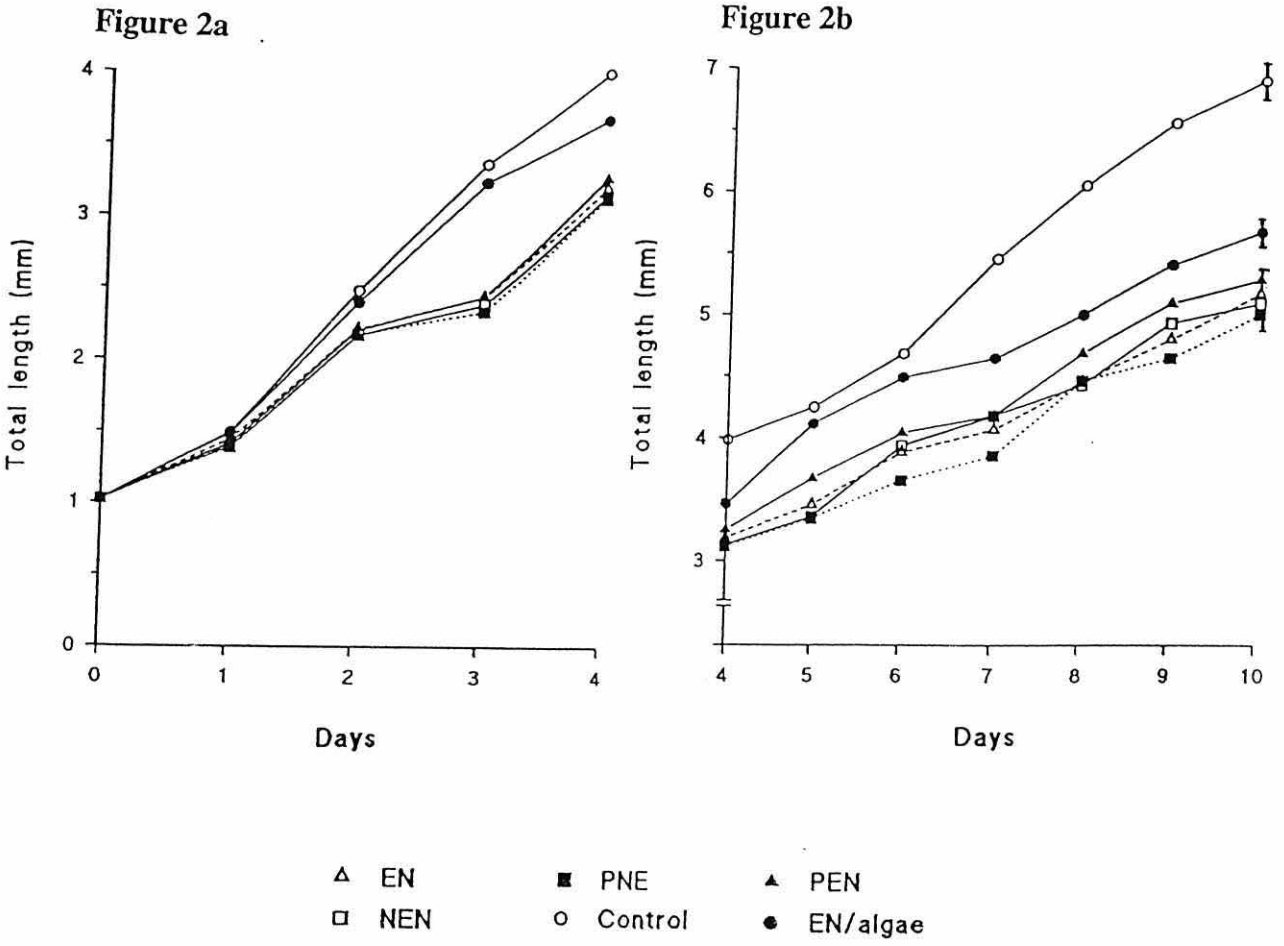


Figure 1. The survival of *P. indicus* larvae (%) reared on various feeding regimes from PZ1 to PL1 stages. EN=lipid enriched, NEN=non enriched, PEN=pigmented and lipid enriched, PNE=pigmented and non enriched nematodes all through larval stages, EN/algae=EN nematodes and 30 cells μl^{-1} algae as a co-feed for 24 h during PZ1 stages, Control=mixed algae during protozoeal stages and plus *Artemia* during later stages. Results are a mean of 3 replicates.



Figures 2a and 2b. The growth (mm) of *P. indicus* larvae reared on feeding regimes (2a) from PZ1 to PZ3/M1 and (2b) from M1 to PL stages. EN=lipid enriched, NEN=non enriched, PEN=pigmented and lipid enriched, PNE=pigmented and non enriched nematodes all through larval stages, EN/algae=EN nematodes and 30 cells μl^{-1} algae as a co-feed for 24 h during PZ1 stages, Control=Mixed algae during protozoeal stages , plus *Artemia* during later stages. Results are a mean of 3 replicates.

Experiment 2

Determination of the effects of nematode lipid enrichment using capelin oil and pigmentation on the growth and survival of *P. indicus* larvae.

Table 11 reveals that on day 4 larvae reared on all single nematode feeds (NEN, EN and PEN) were at PZ3 whereas over 50 % of larvae fed either nematodes /algal co-feeds or algae control diet were at M1. The development of larvae fed PEN nematodes or Nematode/algal co-feed was slightly faster than EN and NEN nematodes at PL1/PL2 stages. Larvae fed on the control diet were at stage PL*4* (73%) on day 10 while larvae fed EN nematodes were between M2 and PL1 stages. On day 7, 8 and 9 larvae fed on the control diet EN/Alga1-3 and PEN nematodes respectively, metamorphosed into PL stages.

Nutritional Composition of the nematode feeds.

Table 13 shows the dry weight % composition of protein, lipid and fatty acid profile for nematodes used in experiment 2. Lipid enrichment of the nematodes increased total lipid content of the PEN nematodes (32.90%) compared to NEN nematodes. However the lipid content of the EN nematodes was lower (9.6%) than NEN nematodes (14.1%). NEN nematodes showed a distinct lack of docosahexaenoic acid (22:6ω-3) whereas lipid enriched nematodes had 1.65% (EN) and 4.45% (PEN). NEN nematodes were richer in ω-6 fatty acids whereas EN and PEN nematodes where richer in ω-3 fatty acids. The ratio of ω-6 /ω-3 of the EN nematodes was 2.20-2.61% whereas this ratio was 20.05% in the NEN nematodes. The total protein content of NEN nematodes was 66% compared to EN nematodes (52.3%) and PEN (44.2%).

Survival

Figures 3 and 5 and Table 7 demonstrate larval survivals on various feeds from PZ1 to PL stages and that all nematode feeding regimes, except EN nematodes promoted significantly better larval survival rate and final survival than those of the control treatment. A significant difference was observed at the 1% level between feeding regimes affecting larval survival from PZ1 to PL stages. A lower mortality rate was observed for larvae fed nematodes with an algal co-feed (EN/Alg1 and EN/Alg2) (3.43 - 4.99 % day $^{-1}$) compared to the control (6.78% day^{-1}) and EN treatments. 68% survival at metamorphosis was found with PEN nematodes, which was the

lowest mortality rate (2.37 % day^{-1}).

EN nematodes caused significantly higher mortality rates (11.09% day^{-1}) and also the lowest final survival (10.33 %) at the PL stages at the 5% level. NEN nematodes resulted in larvae having a lower larval mortality rate (4.14 % day^{-1}) with a final survial of 51% at the PL stage.

Growth

PZ1-PZ3/M1

Figures 9a and 9b show larvae of *P. indicus* fed various feeding regimes. From the results presented in Figure 4a it is clear that the introduction of algae (15-30 cells μl^{-1}) into the culture along with EN nematodes promoted similar larval growth rates to those of the live algal control treatment during protozoeal stages. Table 7 shows comparisons of larval growth rates between day 1 and day 4 and demonstrated that the EN/Alg 1 diet supported the highest larval growth rate (0.93 mm day^{-1}) compared to those obtained on all other diets. PEN nematode fed larvae showed significantly better growth rate (0.57mm day^{-1}) at the 5% level than those fed on EN nematodes (0.54 mm day^{-1}) or NEN nematodes (0.54 mm day^{-1}). Nematode fed larvae exhibited lower growth rates (0.54-0.57 mm day^{-1}) compared to those larvae fed on algae only (0.85 mm day^{-1}) or where algal co-feeds were offered in conjunction with the nematodes (0.83 - 0.93 mm day^{-1}).

M1-PL

Table 7 shows that larvae fed nematodes and algal co-feeds had significantly higher growth rates during the herbivorial stages and displayed significantly lower growth rates (0.30 - 0.32 mm day^{-1}) compared to the control diet (0.60 mm day^{-1}). Nematode diets (NEN, PEN and PNE) gave significantly better growth rates (0.39 mm day^{-1}) than EN nematodes. Larvae fed EN nematodes exhibited a slower growth rate (0.28 mm day^{-1}) than that of (0.36 m day^{-1}) NEN nematode fed larvae. On day 10 comparisons were made of larval growth at the PL stage and this indicated that larvae reared on mixed algae and *Artemia* had significantly greater larval total lengths at the 1% level (7.49 mm total length). Table 7 shows that all other diets promoted similar final total lengths at the 5% level at PL stages apart from EN fed nematodes. Tables 12a and 12b showed growth and survival during larval development of *P. indicus* larvae.

Table 7. Growth rate (mm day^{-1}), mortality rate (% day^{-1}), final survival (%) and total length (mm) of *P. indicus* larvae reared on various nematodes feeding regimes and a control diet from PZ1 to PL stages. Treatments with the same superscripts are not significantly different (P>0.05). Values for surival and growth are means +/- s.d. (n=3).

Diets	Mortality rate (% day^{-1})	Final Survival at PL (%)	Growth rate (mm day^{-1}) PZ1-PZ3/M1	M1-PL	Final growth ar PL (mm)
NEN	4.142^{d}	51.00^{b}+/-5.07	0.535^{e}	0.358^{b}	5.48b+/-0.17
EN	11.092^{a}	10.33^{e}+/-2.25	0.540^{e}	0.276^{d}	4.89^{c}+/-0.13
EN/Alg1	3.434^{d}	62.50^{a}+/-3.28	0.927^{a}	0.299^{d}	5.95^{b}+/-0.14
EN/Alg2	4.992^{c}	47.00^{bc}+/-4.81	0.827^{c}	0.317^{c}	5.87^{b}+/-0.15
EN/Alg3	7.392^{b}	37.67^{c}+/-4.81	0.831^{c}	0.299^{d}	5.63^{b}+/-0.13
PEN	2.367^{e}	68.17^{a}+/-2.02	0.570^{d}	0.386^{b}	5.64b+/-0.10
Control	6.784^{b}	32.00^{d}+/-2.60	0.851^{b}	0.599^{a}	7.49^{a}+/-0.18

Table 8a. Two-way ANOVA with days as a covariate on larval survival of *P. indicus* from PZ1 to PL stages.

Source	DF	Seq SS	Adj MS	F	P	Significant
Diet	6	8190.5	1903.8	10.68	0.000	
Day	1	27497.2	27497.2	925.48	0.000	
Repl(Diet)	14	238.0	217.1	0.52	0.911	
Diet*Day	6	6215.5	6215.5	34.87	0.000	***
Repl*Day(Diet)	14	295.8	295.8	0.71	0.755	
Error	63	1871.8	1871.8			
Total	104	44308.8				

Table 8b. Comparison of larval mortality rates (% day $^{-1}$) of *P. indicus* fed on various diets from PZ1 to PL stages.

Term	Mortality rate (% day^{-1})	Coeff	Stdev	t-value	P	Significant
Constant		98.448	1.248	78.91	0.000	
Day		-11.4429	0.3761	-30.42	0.000	
Day*Diet						
NEN	4.142	3.1595	0.9214	3.43	0.001	***
EN	11.092	-10.7405	0.9214	-11.66	0.000	***
EN/Alg1	3.434	4.5762	0.9214	4.97	0.000	***
EN/Alg2	4.992	1.4595	0.9214	1.58	0.118	Ns
EN/Alg3	7.392	-3.0405	0.9214	-3.30	0.002	**
PEN	2.367	6.7095	0.9214	7.28	0.000	***
Control	6.784	-2.1238	0.9214	-2.31	0.024	*

Table 9a. Two-way ANOVA with days as a covariate on larval growth of *P. indicus* from PZ1 to PZ3/M1 stages.

Source	DF	Seq SS	Adj MS	F	P	Sigmificant
Diet	6	88.214	1.086	4.33	0.000	
Day	1	581.776	578.248	1.4E+04	0.000	
Repl(Diet)	14	0.639	0.286	0.49	0.940	
Diet*Day	6	26.953	27.052	107.82	0.000	***
Repl*Day(Diet)	14	0.668	0.668	1.14	0.317	
Error	858	35.880	35.880			
Total	899	734.130				

Table 9b. Comparisons of larval growth rates (mm day^{-1}) of P. indicus fed various diets from PZ1 to PZ3/M1 stages.

Term	Growth rate (mm day^{-1})	Coeff	Stdev	t-value	P	Significant
Constant		0.81729	0.016920	48.31	0.000	
Day		0.726102	0.006175	117.59	0.000	
Day*Diet						
PEN	0.570	-0.15604	0.01505	-10.37	0.000	***
NEN	0.535	-0.19089	0.01537	-12.42	0.000	***
EN	0.540	-0.18581	0.01512	-12.29	0.000	***
EN/Alg1	0.927	0.20144	0.015101	13.42	0.000	***
EN/Alg2	0.827	0.10100	0.01498	6.74	0.000	***
EN/Alg3	0.831	0.10473	0.01525	6.87	0.000	***
Control	0.851	0.12558	0.01510	8.32	0.000	***

Table 10a. Two-way ANOVA with days as a covariate on larval growth of *P. indicus* (M1-PL stages).

Source	DF	Seq SS	Adj MS	F	P	Sigmificant
Diet	6	256.039	22.705	22.50	0.000	
Day	1	503.425	516.268	3069.17	0.000	
Repl(Diet)	14	4.755	1.810	0.77	0.704	
Diet*Day	6	37.232	37.602	37.36	0.000	***
Repl*Day(Diet)	14	2.464	2.464	1.05	0.404	
Error	1329	223.552	223.552			
Total	1370	1027.466				

Table 10b. Comparison of larval growth rates (mm day^{-1}) of *P. indicus* fed various diets (M1-PL1 stages).

Term	Mortality rate (% day^{-1})	Coeff	Stdev	t-value	P	Significant
Constant		3.74380	0.02518	148.68	0.000	
Day		0.362231	0.00654	55.40	0.000	
Day*Diet						
NEN	0.358	-0.00425	0.01568	-0.27	0.786	Ns
EN	0.276	-0.08656	0.01587	-5.45	0.000	***
EN/Alg1	0.299	-0.06307	0.01569	-4.02	0.000	***
EN/Alg2	0.317	-0.04435	0.01590	-2.79	0.005	**
EN/Alg3	0.299	-0.06253	0.01609	-3.89	0.000	***
PEN	0.386	0.02373	0.01587	1.50	0.135	Ns
Control	0.599	0.23704	0.01700	13.94	0.000	***

Table 11. Percentage larval stages of *P. indicus* reared on various feeding regimes from PZ1 to PL stages.

Diets	Larval stages at PZ3/M1 (%) (Day4)	Larval stages at PL (%) (Day 10)
NEN	100% PZ3	40% M3, 45% PL1, 15% PL2
EN	100% PZ3	11% M2, 60% M3, 23% PL1
EN/Alg1	100% M1	8% M3, 72% PL1, 10% PL2, 10% PL3
EN/Alg2	50% PZ3, 50% M1	7% M3, 73% PL1, 13% PL2, 7% PL3
EN/Alg3	23% PZ3, 77% M1	94% PL1, 6% PL2
PEN	100% PZ3	18% M3, 75% PL1, 5% PL2, 2% PL3
Control	37.5% PZ3, 62.5% M1	8% PL2, 19% PL3, 73% PL4

Table 12a. Survival data (%) for Figures 3 and %. Each value is a mean +/- s.d. (n=3).

Days	NEN	EN	EN/Alg1	EN/Alg2	EN/Alg3	PEN	Control
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00
2	85.00+/-2.18	93.00+/-5.63	88.50+/-2.65	88.00+/-2.18	91.50+/-2.98	88.00+/-2.20	85.83+/-4.54
4	70.00+/-3.12	7800+/-1.61	82.00+/-4.27	74.00+/-3.50	82.67+/-1.52	80.00+/-2.18	64.00+/-2.65
6	59.33+/-7.42	57.17+/-5.48	72.00+/-0.87	64.50+/-2.78	58.83+/-4.01	76.00+/-1.73	47.00+/-3.06
8	55.17+/-2.75	21.00+/-2.84	65.33+/-2.02	56.17+/-1.04	45.40+/-2.65	72.33+/-2.57	36.00+/-2.78
10	51.00+/-5.07	10.33+/-2.25	62.50+/-3.28	47.00+/-4.81	37.67+/-4.81	68.17+/-2.02	32.00+/-2.60

Table 12b. Growth data (mm) for Figures 4 and 6. Each value is a mean +/- s.d. (n=3).

Days	NEN	EN	EN/Alg1	EN/Alg2	EN/Alg3	PEN	Control
0	0.93+/-0.06	0.93+/-0.06	0.93+/-0.06	0.93+/-0.06	0.93+/-0.06	0.93+/-0.06	0.93+/-0.06
1	1.39+/-0.01	1.37+/-0.01	1.53+/-0.01	1.42+/-0.01	1.5+/-0.01	1.37+/-0.02	1.53+/-0.02
2	2.13+/-0.01	2.14+/-0.01	2.58+/-0.05	2.45+/-0.06	2.5+/-40.03	2.22+/-0.01	2.63+/-0.05
3	2.46+/-0.01	2.3+/-50.04	3.52+/-0.05	3.47+/-0.01	3.46+/-0.03	2.48+/-0.03	3.47+/-0.02
4	3.08+/-0.13	3.12+/-0.01	4.30+/-0.04	3.88+/-0.14	3.99+/-0.08	3.20+/-0.08	4.09+/-0.10
5	3.71+/-0.14	3.52+/-0.06	4.54+/-0.03	4.25+/-0.07	4.11+/-0.02	3.72+/-0.07	4.34+/-0.18
6	4.27+/-0.05	3.92+/-0.06	4.85+/-0.10	4.79+/-0.03	4.61+/-0.10	4.41+/-0.02	5.09+/-0.07
7	4.53+/-0.07	4.23+/-0.16	5.15+/-0.16	5.02+/-0.05	4.79+/-0.04	4.57+/-0.14	5.54+/-0.06
8	5.07+/-0.13	4.58+/-0.15	5.43+/-0.07	5.33+/-0.16	5.00+/-0.10	5.13+/-0.05	6.06+/-0.16
9	5.34+/-0.15	4.78+/-0.05	5.61+/-0.12	5.69+/-0.14	5.54+/-0.04	5.53+/-0.10	6.88+/-0.14
10	5.48+/-0.17	4.89+/-0.13	5.95+/-0.14	5.87+/-0.15	5.63+/-0.13	5.64+/-0.10	7.49+/-0.18

Table 13. The nutritional profile of nematode diets fed to *P. indicus* larvae from PZ1 to PL stages. NEN = non-enriched, EN= lipid enriched, PEN = pigmented and lipid enriched nematodes.

	NEN	EN	PEN
Protein %	66.00	52.30	44.20
Lipid %	14.10	9.60	32.90
Fatty acids			
14:0	0.2	0.52	2.27
16:0	5.18	3.52	8.37
16:1ω-9	0.17	0.38	0.33
16:1ω-7	0.65	1.76	2.97
18:0	7.2	6.58	2.85
18:1ω-9	19.61	10.2	16.04
18:1ω-7	2.78	4.39	2.53
18:2ω-6	34.88	26.11	26.52
18:3ω-3	0.43	0.4	0.8
18:4ω-3	0.17	0.25	1.33
20:0	0.5	0.27	0.22
20:1ω-9	0.66	4.06	6.27
20:2ω-6	1.61	1.89	0.65
20:3ω-6	5.69	3.79	0.97
20:4ω-6	9.34	6.41	1.79
20:4ω-3		1.23	0.71
20:5ω-3	1.97	10.99	5.85
22:1ω-11	-	3.09	7.62
22:5ω-3	-	0.14	0.45
22:6ω-3	-	1.65	4.48
Saturates %	13.08	10.89	13.71
Monosaturates %	23.87	23.88	35.76
Polysaturates %	54.09	52.86	43.55
Sum %	91.04	87.63	93.02
ω-3	2.57	14.66	13.62
ω-6	51.52	38.20	29.93
ratio ω-6/ω-3	20.05	2.61	2.20

Figure 3

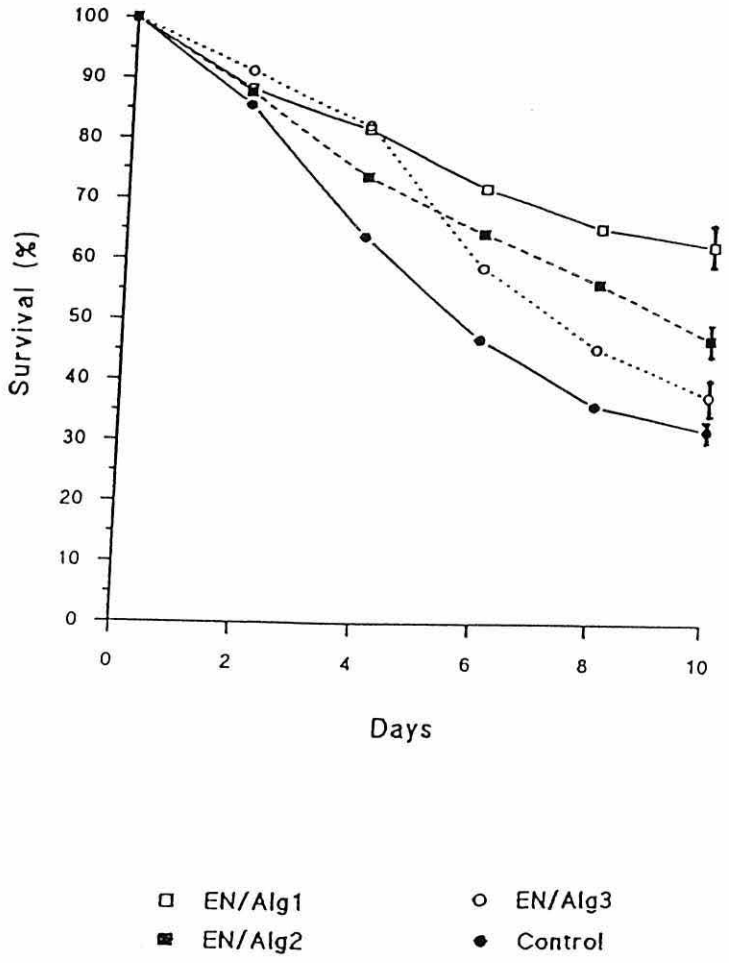


Figure 3. The survival of *P. indicus* larvae (%) reared on various feeding regimes from PZ1 to PL1 stages. EN/algae1=lipid enriched nematodes and 30 cells μl^{-1} algae as a co-feed for 24 h during PZ1 stages, EN/algae2=EN nematodes and 30 cells μl^{-1} algae as a co-feed for 48 h during PZ1 stages, EN/algae3=EN nematodes and 15 cells μl^{-1} algae as a co-feed for 48 h during PZ1 stages, Control=mixed algae during protozoeal stages and plus *Artemia* during later stages. Results are a mean of 3 replicates

Figure 4a

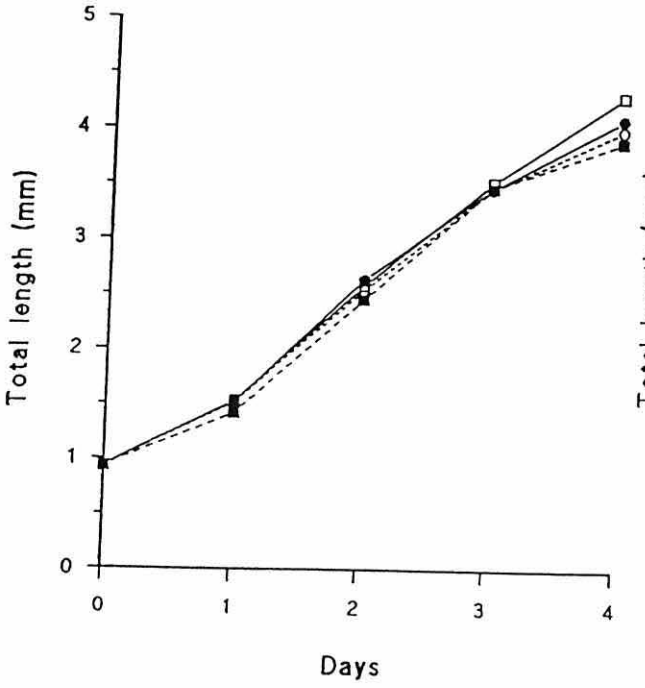
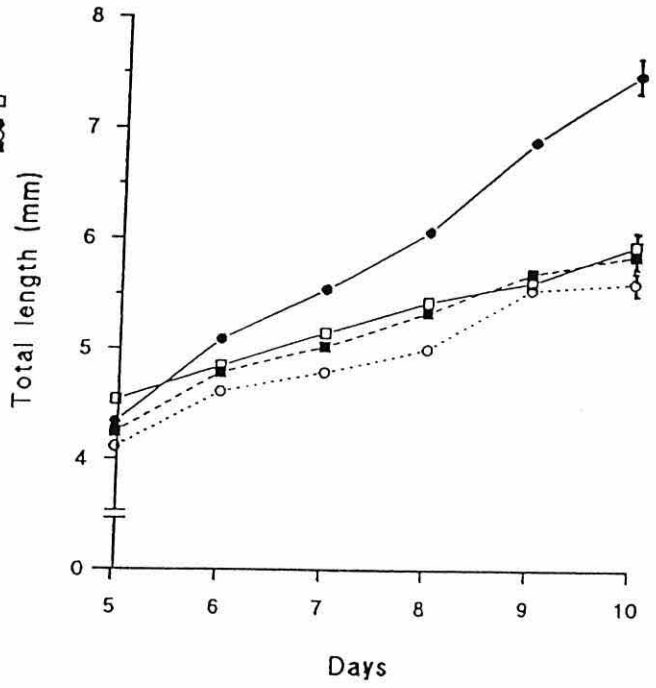


Figure 4b



Figures 4a and b. The growth (mm) of *P. indicus* larvae reared on feeding regimes (4a) from PZ1 to PZ3/M1 and (4b) from M1 to PL stages. 1 EN/algae=lipid enriched nematodes and 30 cells μl^{-1} algae as a co-feed for 24 h during PZ1 stages, 2 EN/algae=EN nematodes and 30 cells μl^{-1} algae as a co-feed for 48 h during PZ1 stages, 3 EN/algae=EN nematodes and 15 cells μl^{-1} algae as a co-feed for 48 h during PZ1 stages, Control=mixed algae during protozoeal stages and plus *Artemia* during later stages. Results are a mean of 3 replicates

Figure 5

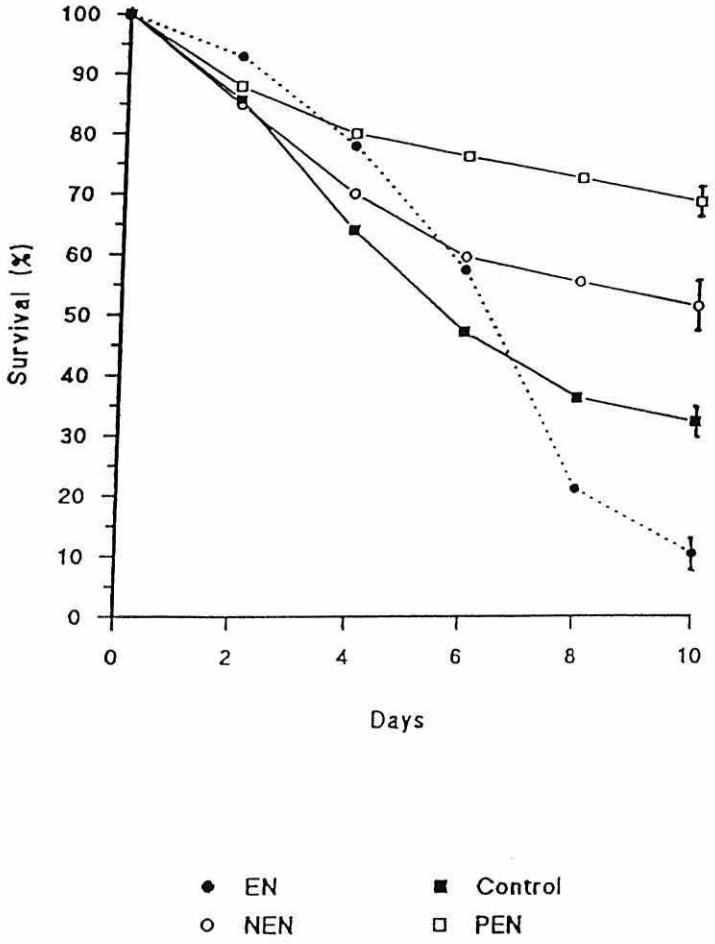


Figure 5. The survival of *P. indicus* larvae (%) reared on various feeding regimes from PZ1 to PL1 stages. EN=lipid enriched, NEN=non enriched, PEN=pigmented and enriched nematodes, Control=mixed algae during protozoeal stages and plus *Artemia* during later stages. Results are a mean of 3 replicates.

Figure 6a

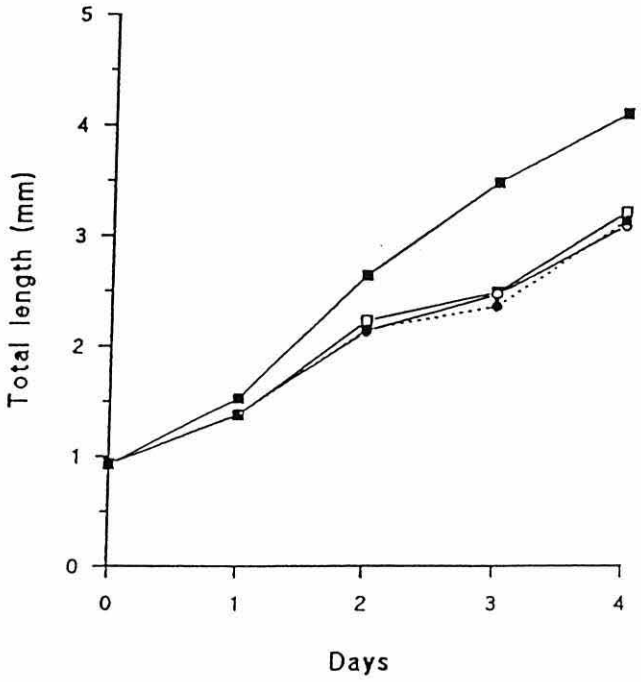
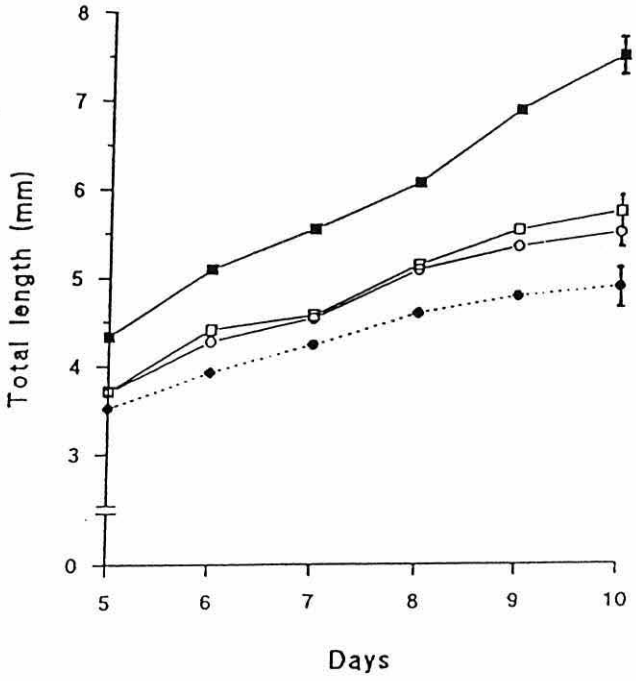


Figure 6b



Figures 6a and b. The growth (mm) of *P. indicus* larvae reared on feeding regimes (6a) from PZ1 to PZ3/M1 and (6b) from M1 to PL stages. EN=lipid enriched, NEN=non enriched, PEN=pigmented and enriched nematodes,Control=mixed algae during protozoeal stages and plus *Artemia* during later stages. Results are a mean of 3 replicates.

Experiment 3

The effects of nematodes (*P. redivivus*) enriched with different lipid sources on larval growth and survival of *P. indicus*

Table 15 shows that cod liver oil enriched nematodes (CLO) and lipid enriched (EN) nematodes fed larvae and controls developed to M1 a day earlier than larvae fed MAR and NEN nematodes. Larvae fed on the lipid enriched nematodes metamorphosed into PL1 stage 1 day earlier than those fed on NEN nematodes. It was found however that larvae fed the control treatments had the shortest duration until metamorphosis of 8 days.

Nutritional composition of nematode feeds.

Tables 21a and 21b show the nutritional composition and fatty acid profile of the nematodes used during experiment 3. All the lipid enriched nematodes had a higher total lipid content than NEN nematodes. Lipid enriched nematodes contained substantially higher levels of ω-3 PUFA (10.62-14.9%) than NEN nematodes (2.21-2.94%). Lipid enriched nematodes were richer in 22:5ω-3 fatty acids (5.17 - 6.83%) than NEN (1.29 - 1.42%). The percentage level of 22:6ω-3 was also higher in the nematodes enriched with lipids (EN, 3.91-4.0%, MAR, 3.74-4.39% and CLO, 2.79-2.86%) compared to NEN nematodes (0-0.8 %). The level of ω-6 series in lipid enriched nematodes was comparable to that of NEN nematodes. The protein content of NEN nematodes (47.4% -77%) was higher than in the EN nematodes (52.9-64.6%). The results presented in Table 21a and 21b demonstrate that the nutritional content of fatty acid profile of these nematode feeds larvae did not vary a great deal.

Survival

PZ1 - PZ3/M1

Figure 8a shows the survival of *P. indicus* larvae reared on various feeding regimes between PZ1 and PZ3/M1 stages. Tables 16a and 16b show that there were signficant differences between the effects of diets on larval survival during these stages. Survival of larvae at M1 showed that all lipid enriched nematodes had significantly higher survivals at the 5% level (73-88%). The highest mortality rates were found in larvae fed the NEN nematodes (7.55% day^{-1}). CLO

nematodes promoted the highest survival until M1 stage however there was no significant difference at the 5% level between PZ1 and M1 stages.

M1-PL1

Table 20a summarises data shown in Figures 8a and 8b. Figure 8b shows survival of the larvae fed various diets between M1 to PL stages. Tables 17a and 17b demonstrate that there was a significant effect on larval growth for larvae fed the various feeding regimes at the 5% level. Figure 10b shows that larval mortality declined during mysis stages. Table 15 shows that there was no significant difference at the 5% level between survival for larvae fed various lipid enriched nematodes. When comparing the survival at the PL1 stage the results show that NEN nematodes and the control diets resulted in significantly lower survivals compared to other nematode feeding regimes at the 5% level.

Growth

PZ1-PZ3/M1

Figure 9a shows that growth rates of larvae fed various feeds during protozoeal stages, were signficantly different (Table 18a and 18b). Table 14 demonstrates that the highest growth rate was achieved with EN nematodes (0.574 mm day^{-1}) and the control diet (0.576 mm day^{-1}). Table 15 shows that growth rate of larvae fed MAR and NEN nematodes were not significantly different and comparisons of larval growth rate at M1 stage using one-way ANOVA demonstrate that EN nematodes and the control algae promoted the greatest total length (4.37 and 4.35 mm total length) compared with other nematode feeds at the 5% level.

M1-PL1

Table 20b summarises growth data for figures 9a and 9b. Figure 9b shows the growth of the larvae during mysis and early postlarval stages. In Table 19a and 19b comparisons of larval growth rates between M1 and PL stages showed that MAR nematodes gave the highest growth rate (0.425 mm day^{-1}) compared to all other lipid enriched nematodes and the control diet (0.360 mm day^{-1}). Table 15 shows the significant difference at the 1% level in total length of PL1 larvae fed on different diets. Larvae fed CLO nematodes displayed a higher larval growth rate (0.273 mm day^{-1}) than EN nematode fed larvae (0.234 mm day^{-1}). The control treatments and MAR

nematode fed larvae supported the highest mean total length (5.71 and 5.66 mm) respectively. There was a significant difference at the 5% level of the total length between CLO nematodes (5.37 mm) and NEN (5.28 mm).

Table 14. Comparisons of mortality rate (% day^{-1}) and growth rate (mm day^{-1}) of *P. indicus* larvae fed various feeding regimes. Values with the same superscripts are not significantly different (P>0.05).

Diets	Mortality rate (% day^{-1})		Growth rate (mm day^{-1})	
	PZ1-PZ3/M1	M1-PL1	PZ1-PZ3/M1	M1-PL1
MAR	5.402^{b}	1.067	0.516^{c}	0.425^{a}
NEN	7.552^{a}	2.717	0.523^{c}	0.236^{d}
CLO	2.300^{d}	1.800	0.536^{b}	0.273^{c}
EN	3.967^{c}	2.450	0.574^{a}	0.234^{d}
Control	6.560^{a}	3.100	0.576^{a}	0.360^{b}

Table 15. Survival, total length and duration of larval development of *P. indicus* larvae at M1 and PL1 stages in various diets. Values with different superscripts are significantly different (P<0.05). Values are means +/- s.d.(n=3).

Diets	Total length (mm)		Survival (%)		Duration (days)	
	M1	PL1	M1	PL1	to M1	to PL1
MAR	4.18^{b}+/-0.06	5.66^{ab}+/-0.09	73.33^{ab}+/-4.15	69.00^{a}+/-5.29	5	9
NEN	4.19^{b}+/-0.02	5.28^{c}+/-0.08	60.83^{c}+/-4.31	53.50^{b}+/-5.50	5	10
CLO	4.23^{a}+/-0.03	5.37^{c}+/-0.05	87.83^{a}+/-2.36	77.00^{a}+/-5.57	4-5	9-10
EN	4.38^{a}+/-0.03	5.53^{b}+/-0.03	78.33^{a}+/-4.31	71.50^{a}+/-3.91	4-5	9-10
Control	4.37^{a}+/-0.02	5.71^{a}+/-0.05	67.75^{b}+/-3.25	54.83^{b}+/-4.25	4-5	8-9

Table 16a. Two-way ANOVA with days as a covariate on larval survival (%) of *P. indicus* from PZ1 to PZ3/M1 stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Sigmificant
Diet	4	2369.68	147.55	36.89	3.55	0.012	
Day	1	6804.3	6644.12	6644.12	639.35	0	
Repl(Diet)	10	280.35	132.47	13.25	1.27	0.266	
Diet*Day	4	918.59	911.35	227.84	21.92	0	***
Repl*Day(Diet)	10	147.9	147.90	14.79	1.42	0.193	
Error	59	613.13	613.13	10.39			
Total	88	11133.94					

Table 16b Comparison of larval mortality rates (% day^{-1}) of *P. indicus* fed various diets from PZ1 to PZ3/M1.

Term	Mortality rate (% day^{-1})	Coeff	Stdev	t-value	P	Significant
Constant		104.117	0.802	129.79	0.000	
Day		-5.1552	0.2039	-25.29	0.000	
Day*Diet						
MAR	5.402	-0.2471	0.4359	-0.57	0.573	Ns
NEN	7.552	-2.3971	0.4004	-5.99	0.000	***
CLO	2.300	2.8600	0.4004	7.14	0.000	***
EN	3.967	1.1886	0.4004	2.97	0.004	**
Algae	6.560	-1.4043	0.4004	-3.51	0.001	***

Table 17a. Two-way ANOVA with days as a covariate on larval survival (%) of *P. indicus* between M1 and PL stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Sigmificant
Diet	4	6397.36	193.63	48.41	2.74	0.040	
Day	1	685.91	697.22	697.22	39.53	0.000	
Repl(Diet)	10	361.43	289.51	28.95	1.64	0.126	
Diet*Day	4	125.88	66.79	16.70	0.95	0.446	Ns
Repl*Day(Diet)	10	290.43	290.43	29.04	1.65	0.125	
Error	44	775.97	775.97	17.64			
Total	73	8636.98					

Table 17b. Conparison of larval mortality rates (% day $^{-1}$) of *P. indicus* fed various feeding regimes between M1 and PL stages.

Term	Mortality (% day^{-1})	Coeff	Stdev	T-value	P	Significant
Constant		86.767	2.897	29.95	0.000	
Days		-2.2267	0.3541	-6.29	0.000	
Days*Diets						
MAR	1.067	1.1600	0.7718	1.50	0.140	Ns
NEN	2.717	-0.4900	0.6915	-0.71	0.482	Ns
CLO	1.800	0.4267	0.6915	0.62	0.540	Ns
EN	2.450	-0.2233	0.6915	-0.32	0.748	Ns
Control	3.100	-0.8733	0.6915	-1.26	0.213	Ns

Table 18a. Two-way ANOVA with days as a covariate on larval growth (mm TL) of *P. indicus* from PZ1 to PZ3/M1 stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Sigmificant
Diet	4	3.121	0.576	0.144	3.38	0.009	
Day	1	847.713	847.347	847.347	2.0E+04	0.000	
Repl(Diet)	10	0.203	0.057	0.006	0.13	0.999	
Diet*Day	4	1.851	1.846	0.462	10.85	0.000	***
Repl*Day(Diet)	10	0.081	0.081	0.008	0.19	0.997	
Error	948	40.320	40.320	0.043			
Total	977	893.289					

Table 18b. Comparison of larval growth rates (mm day^{-1}) of *P. indicus* fed on various feeding regimesfrom PZ1 to PZ3/M1 stages.

Term	Growth rate (mm day^{-1})	Coeff	Stdev	t-value	P	Significant
Constant		0.88626	0.01533	57.80	0.000	
Day		0.545019	0.003861	141.15	0.000	
Day*Diet						
MAR	0.516	-0.029436	0.007710	-3.82	0.000	***
NEN	0.523	-0.021858	0.007629	-2.87	0.004	**
CLO	0.536	-0.021858	0.007793	-1.12	0.261	Ns
EN	0.574	0.028737	0.007762	3.70	0.000	***
Algae	0.576	0.031317	0.007718	4.06	0.000	***

Table 19a. Two-way ANOVA with days as a covariate on larval growth of *P. indicus* (M1-PL stages).

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Sigmificant
Diet	4	31.2475	5.3624	1.3406	15.32	0.000	
Day	1	170.9386	168.9534	168.9534	1930.75	0.000	
Repl(Diet)	10	2.2207	1.1371	0.1371	1.57	0.111	
Diet*Day	4	10.2721	10.2672	2.5668	29.33	0.000	***
Repl*Day(Diet)	10	2.8645	2.8645	0.2865	3.27	0.0000	
Error	889	77.7934	77.7934	0.0875			
Total	918	295.3368					

Table 19b. Comparison of larval growth rates (mm day^{-1}) of *P. indicus* reared on various feeding regimes (M1-PL stages).

Term	Growth rate (mm day^{-1})	Coeff	Stdev	T-value	P	Significant
Constant		3.95289	0.02326	169.93	0.000	
Days		0.305616	0.00696	43.94	0.000	
Days*Diets						
MAR	0.425	0.11915	0.01382	8.62	0.000	***
NEN	0.236	-0.06915	0.01350	-5.12	0.000	***
CLO	0.273	-0.03276	0.01386	-2.36	0.018	*
EN	0.234	-0.07190	0.01443	-4.98	0.000	***
Control	0.360	0.05465	0.01393	3.92	0.000	***

Table 20a. Data for Figures 8a and 8b. Each value represents a mean +/- s.d. (n=3).

Days	MAR	NEN	CLO	EN	CONTROL
0	100.00	100.00	100.00	100.00	100.00
1	98.67+/-2.31	97.50+/-2.29	100+/-0.00	96.83+/-1.04	100.00
2	94.33+/-0.29	91.67+/-5.39	97.50+/-1.80	94.17+/-1.04	97.17+/-2.75
3	90.17+/-2.25	77.17+/-3.69	93.83+/-3.69	87.67+/-2.75	91.67+/-4.75
4	82.50+/-2.00	72.83+/-6.25	93.33+/-4.31	83.33+/-1.61	83.83+/-3.01
5	75.17+/-6.75	63.33+/-2.56	91.17+/-4.31	80.1+/-2.89	77.00+/-4.50
6	73.33+/-4.15	60.83+/-4.31	87.83+/-2.36	78.33+/-4.31	67.75+/-3.25
7	72.17+/-6.79	59.67+/-7.29	84.83+/-6.25	76.83+/-4.16	61.67+/-7.09
8	71.17+/-5.79	56.67+/-6.29	80.17+/-3.75	75.17+/-2.47	57.25+/-5.25
9	70.17+/-5.62	53.83+/-7.64	79.33+/-6.43	72.50+/-4.36	56.50+/-3.50
10	69.00+/-5.29	53.50+/-5.50	77.00+/-5.57	71.59+/-3.91	54.83+/-4.25

Table 20b. Data for Figures 8a and 8b. Each value is a mean +/- s.d. (n=3)

Days	MAR	NEN	CLO	EN	CONTROL
0	1.02+/-0.03	1.02+/-0.03	1.02+/-0.03	1.02+/-0.03	1.02+/-0.03
1	1.33+/-0.01	1.31+/-0.01	1.33+/-0.02	1.31+/-0.01	1.32+/-0.01
2	2.21+/-0.01	2.14+/-0.02	2.17+/-0.01	2.16+/-0.04	2.22+/-0.01
3	2.47+/-0.03	2.38+/-0.04	2.40+/-0.02	2.35+/-0.07	2.53+/-0.06
4	3.23+/-0.05	3.11+/-0.06	3.14+/-0.07	3.23+/-0.01	3.41+/-0.02
5	3.28+/-0.03	3.19+/-0.07	3.34+/-0.07	3.40+/-0.08	3.54+/-0.06
6	4.13+/-0.03	4.16+/-0.01	4.22+/-0.05	4.34+/-0.05	4.38+/-0.03
7	4.53+/-0.25	4.79+/-0.25	4.39+/-0.03	4.37+/-0.09	4.92+/-0.08
8	4.76+/-0.08	4.83+/-0.04	4.62+/-0.03	4.66+/-0.08	5.06+/-0.13
9	5.23+/-0.13	5.22+/-0.10	4.99+/-0.09	4.98+/-0.02	5.56+/-0.18
10	5.97+/-0.21	5.33+/-0.03	5.06+/-0.06	5.28+/-0.07	5.86+/-0.24

Table 21a The nutritional profiles of nematodes used in the first week of this experiment to feed *P. indicus* larvae from PZ1 to PL stages. NEN=non-enriched, EN=capelin-enriched, MAR=marilla lipid enriched and CLO cod liver lipid-enriched.

	NEN	EN	MAR	CLO
Protein %	74.4	64.6	55.4	56.7
Lipid %	18.5	22.0	20.1	23.0
Fatty acids				
14:0	0.21	1.76	1.01	1.14
16:0	8.21	8.46	4.62	7.33
16:1ω-9	0.27	0.36	0.32	0.32
16:1ω-7	0.82	2.51	3.54	2.67
18:0	5.66	3.02	2.94	3.18
18:1ω-9	22.37	15.74	17.59	17.19
18:1ω-7	3.59	3.35	4.25	3.87
18:2ω-6	29.44	27.51	29.40	29.81
18:3ω-3	0.66	0.98	1.1	0.89
18:4ω-3	0.19	1.04	1.04	0.71
20:0	0.77	0.32	0.23	0.35
20:1ω-9	0.84	4.61	5.26	4.79
20:2ω-6	1.28	0.63	0.99	0.8
20:3ω-6	2.96	0.69	1.21	1.03
20:4ω-6	5.97	1.62	2.31	1.93
20:4ω-3		0.61	0.61	0.43
20:5ω-3	1.29	5.24	6.0	5.33
22:1ω-11	-	0.19	2.19	2.79
22:5ω-3	-	0.39	1.32	0.47
22:6ω-3	0.8	4.0	4.39	2.79
Saturates %	14.85	13.59	8.84	12.0
Monosaturates %	27.89	26.76	33.15	31.15
Polysaturates %	42.59	42.71	48.37	44.19
Sum %	85.33	83.06	90.36	87.82
ω-3	2.94	12.26	14.46	10.62
ω-6	39.65	30.45	33.91	33.57
ratio ω-6/ω-3	13.49	2.48	2.35	3.16

Table 21b. The nutritional profiles of nematodes used in the second week of this experiment to feed *P. indicus* larvae from PZ1 to PL stages. NEN=non-enriched, EN=capelin-enriched, MAR=marilla lipid enriched and CLO cod liver lipid-enriched.

	NEN	EN	MAR	CLO
Protein %	77.0	52.9	59.1	51.9
Lipid %	14.8	16.9	19.9	18.8
Fatty acids				
14:0	0.27	1.96	0.81	1.17
16:0	6.77	8.22	3.68	7.61
16:1ω-9	0.46	0.8	0.33	0.28
16:1ω-7	0.88	2.7	2.92	2.84
18:0	5.11	2.78	2.51	3.4
18:1ω-9	18.61	18.14	5.62	17.57
18:1ω-7	3.9		8.54	4.42
18:2ω-6	24.65	25.17	22.29	26.87
18:3ω-3	0.56	0.99	0.75	0.92
18:4ω-3	0.23	1.15	0.85	0.65
20:0		0.2	0.25	0.19
20:1ω-9	0.91	4.7	4.69	4.81
20:2ω-6	1.2	0.63	1.1	0.68
20:3ω-6	3.29	0.68	1.82	0.85
20:4ω-6	8.59	1.54	2.58	1.87
20:4ω-3		0.24	1.82	0.5
20:5ω-3	1.42	5.17	6.83	5.73
22:1ω-11	-	7.25	1.09	0.51
22:5ω-3	-	0.41	1.41	0.62
22:6ω-3	-	3.91	3.74	2.86
Saturates %	12.15	13.16	7.25	12.37
Monosaturates %	24.76	33.59	23.19	30.43
Polysaturates %	39.94	39.89	42.69	41.55
Sum %	76.85	86.64	73.13	84.35
ω-3	2.21	11.87	14.9	11.28
ω-6	37.73	28.02	27.79	30.27
ratio ω-6/ω-3	17.07	2.36	1.87	2.68

Figure 7a

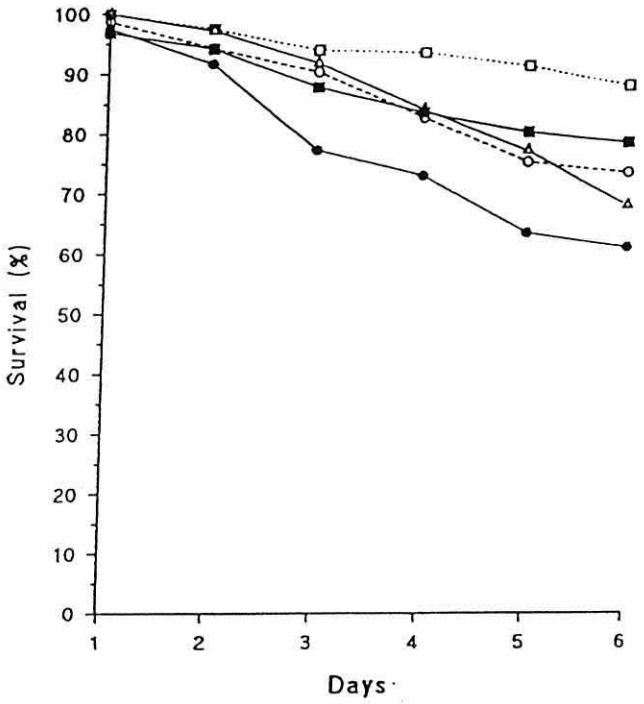
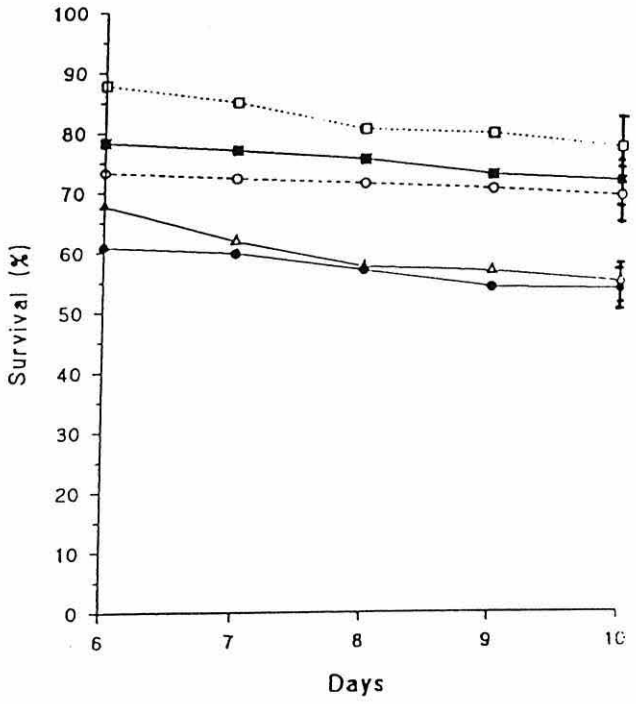


Figure 7b



Figures 7a and b. The survival (%) of *P. indicus* larvae reared on feeding regimes (7a) from PZ1 to PZ3/M1 and (7b) from M1 to PL stages. MAR=marilla lipid enriched, NEN non enriched, CLO=cod liver lipid enriched, EN=capelin lipid enriched, and control= mixed algae during protozoeal stages and plus *Artemia* during later stages. Results are a mean of 3 replicates.

Figure 8a

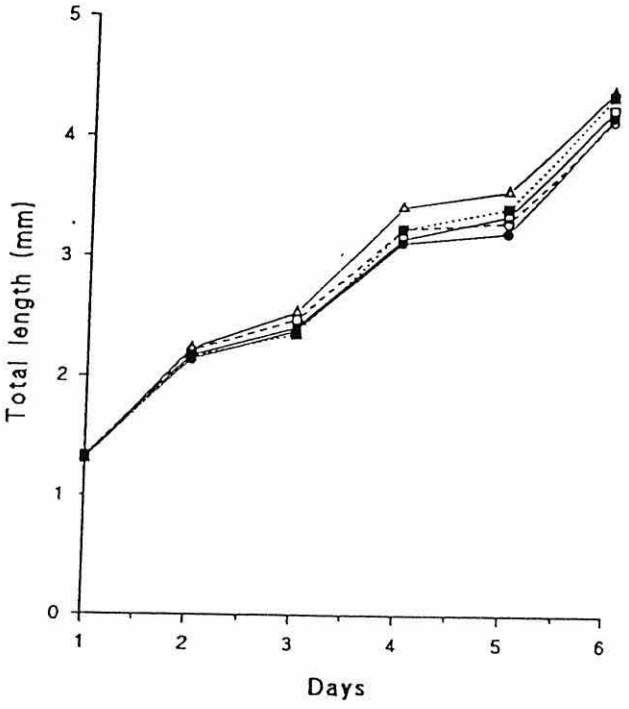
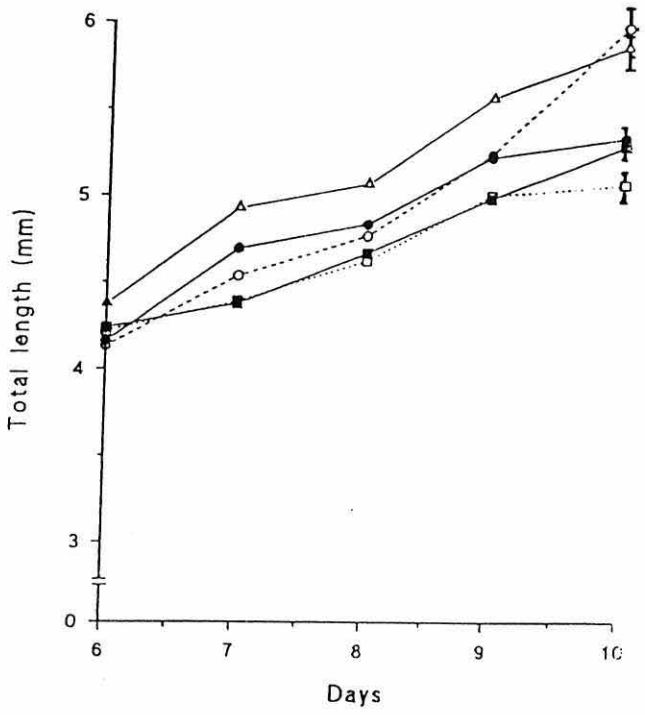
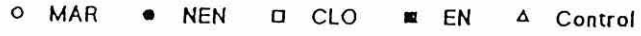


Figure 8b





Figures 8a and 8b. The growth (mm) of *P. indicus* larvae reared on feeding regimes (8a) from PZ1 to PZ3/M1 and (8b) from M1 to PL stages. MAR=marilla lipid enriched, NEN non enriched, CLO=cod liver lipid enriched, EN=capelin lipid enriched, and control= mixed algae during protozoeal stages and plus *Artemia* during later stages. Results are a mean of 3 replicates.

Experiment 4

The effects of nematodes (*P. redivivus*) enriched with either pigment or a placebo on *P. indicus* larvae.

Table 23 shows larval development to M1 and PL stages. Larvae fed a live mixed algae reached M1 stage on day 5. However there were more than 50% on day 6. Pigmented enriched nematodes (PEN) and cod liver oil enriched (CLO) fed larvae started to develop into M1 stage one day earlier (day 6) than those fed placebo enriched nematodes (PLC) (day7). On day 9 larvae fed PEN and CLO nematodes metamorphosed into PL1 stage whilst PLC nematode fed larvae reached this stage on day 10. Larvae fed control diets metamorphosed 1-2 days earlier.

Nutritional composition of nematode feeds.

Tables 29a and 29b shows the total protein, lipid content and fatty acid of the nematodes used to feed *P. indicus* larvae in this experiment. Total lipid content of the PEN, PLC and CLO enriched nematodes were 22.8-32%, 34.6-35.2% and 31.9-38.5% respectively. The ω-3 (PUFA) fatty acid content of PLC nematodes was lower (10.37-12.44%) in comparison to PEN (14.7-15.28%) and CLO nematodes (13.85-34.16%). However ω-6 series (PUFA) of the PLC nematodes (31.85-34.16%) was similar to that of PEN (24.5- 29.97%) and CLO (27.58-28.19%). Comparable amounts of 20:5ω-3 were found in the PLC nematodes (4.49-7.96%). PEN (7.59-9.01%) and CLO (6.04-9.22%). Comparable amounts of 22:6ω-3 were also found in the PLC nematodes (2.57-3.22%), PEN (4.02-4.1%) and CLO (4.24- 5.24%). Total protein content of the nematodes PLC, PEN and CLO were 55.6-59.6%, 51.8-54.9 and 53.9-54% respectively.

Survival

PZ1-PZ3/M1

Figure 10a shows survival of *P. indicus* larvae fed various feeding regimes during protozoeal stages. Tables 24a and 24b show the larval mortality rates compared between day 3 and day 6. Table 22 shows the lowest mortality rate was achieved by larvae fed PEN nematodes (2.83%) and the highest mortality rate was found in larvae fed live mixed algae during protozoeal stages (17.68% day^{-1}). At M1 stage larval total lengths were compared and it was demonstrated that that survival (91%) of larvae on PEN nematodes at M1 stage was not significantly different at the 5%

level than those fed on CLO nematodes (88%)or PLC nematodes (80%) (Table 23). The control treatment resulted in only 33% survival for PZ1 to M1 stage. The PLC nematode and CLO nematode fed larvae showed significantly higher mortality rates (4.22 -6.30 day^{-1}) than PEN nematode fed larvae at the 1% level.

Table 22. Comparisons of mortality rate (% day^{-1}) and growth rate (mm day^{-1}) of *P. indicus* larvae fed various feeding regimes. Values with the same superscripts are not significantly different (P>0.05). Pigmented enriched nematodes (PEN), placebo enriched nematodes (PLC), cod liver oil enriched (CLO). Control = live mixed algae (25 cells μl^{-1} *T. chuii*/45 cells μl^{-1} *S. costatum*) and five *Artemia* ml^{-1} during mysis and PL stages.

Diets	Mortality rate (% day^{-1})		Growth rate (mm day^{-1})	
	PZ1-PZ3/M1	M1-PL1	PZ1-PZ3/M1	M1-PL1
PLC	6.300^{b}	0.717	0.415^{d}	0.518^{b}
PEN	2.833^{d}	1.033	0.455^{b}	0.527^{b}
CLO	4.217^{c}	3.267	0.440^{c}	0.559^{a}
Control	17.675^{a}	-	0.589^{a}	0.465^{c}

M1-PL1

The survival data did not show a linear relationship and it was therefore excluded from the calculations (Figure 10b). Tables 25a and 25b showed no significant difference at the 5% level of larval survival when comparisons were made of mortality rates between M1 and PL1 stages. Table 23 shows no significant differences were found in the final survival of larvae fed the 3 types of nematodes from PZ1 to PL1 stages. Table 22 also demonstrates that all the nematode feeds results in low mortality rates of between 0.72 and 3.27%. Survivals of 88%, 79% and 78% were obtained from larvae (PZ1 fed PEN, CLO and PLC nematodes respectively. Only 11% of the larvae fed the control feeds developed into PL1 stage. Table 28a summarises survival data for figure 10a and 10b.

Table 23. Survival, total length and duration of *P.indicus* larvae at M1 and PL1 stages on various diets. Values with different superscripts are significantly different (P<0.05). Values are means +/- s.d. (n=3).

Diets	Survival (%)		Total length (mm)		Duration (days)	
	M1	PL1	M1	PL1	to M1	to PL1
PLC	79.83^{a}+/-3.18	77.83^{a}+/-3.55	3.77^{c}+/-0.03	5.03^{b}+/-0.04	7	10
PEN	90.67^{a}+/-2.75	87.67^{a}+/-2.32	3.88^{b}+/-0.03	5.18^{a}+/-0.08	6-7	9-10
CLO	87.63^{a}+/-5.89	79.17^{a}+/-7.01	3.79^{c}+/-0.02	5.13^{ab}+/-0.08	6-7	9-10
Control	33.17^{b}+/-4.04	10.50^{b}+/-4.71	3.92^{a}+/-0.02	5.23^{a}+/-0.05	5-6	8-9

Growth

PZ1-PZ3/M1

Table 22 demonstrates that the control diet supported the highest larval growth rate (0.89 mm day^{-1}) and the growth rates of larvae fed the nematode feeds were significantly different at the 1% level from all other diets (day 1-day 6) as shown in Table 26a and 26b. The larvae fed on PEN nematodes gave a higher growth rate (0.455 mm day^{-1}) than PLC nematodes (0.415 mm day^{-1}) and CLO nematodes (0.440 mm day^{-1}). Table 23 shows that comparisons of larval total lengths at M1 stage also indicated the same result in that PEN nematodes fed larvae had significantly greater lengths (3.88mm total length) at the 5% level compared to those fed either PLC nematodes (3.77mm total length) or CLO nematodes (3.79mm total length). It was the live mixed algae diet that promoted significantly better total lengths (3.92 mm). Figure 11a shows growth of the larvae fed various feeding regimes from PZ1 to PZ3/M1 stages.

M1-PL1

Tables 27a and 27b show that the growth rates of larvae were significantly affected at the 1% level by feeds. The highest larval growth was obtained from larvae fed CLO nematodes (0.559 mm day^{-1}) as shown in Table 22. The larval growth rate on PEN nematodes and PLC nematodes did not differ significantly at the 5% level during this period. The control diet produced larvae with the lowest growth rate (0.465 mm day^{-1}). Table 23 shows the final total length on day 10 of larvae fed PEN nematodes (5.18mm), these results were signficantly different at the 5% level and greater than for larvae fed PLC diet (5.03 mm). Larvae fed on the control diet reached a length of 5.23 mm at PL1 stage. Figure 11b shows growth of the larvae between M1 and PL stages and the growth data is summarised in Table 28b.

Table 24a. Two-way ANOVA with day as a covariate on survival of *P. indicus* larvae from PZ2 to PZ3/M1 (day3 to day 6) on various feeding regimes.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Sigmificant
Diet	3	5819.27	553.67	184.56	16.29	0.000	
Day	1	4686.08	4686.08	4686.08	413.67	0.000	
Repl(Diet)	8	253.13	12.33	1.54	0.14	0.997	
Diet*Day	3	3556.39	3556.39	1185.46	104.65	0.000	***
Repl*Day(Diet)	8	71.34	71.34	8.92	0.79	0.619	
Error	24	271.87	271.87	11.33			
Total	47	14658.08					

Table 24b. Comparison of larval survival rates (% day^{-1}) from PZ2-PZ3/M1 stages.

Term	Mortality rate (% day^{-1})	Coeff	Stdev	t-value	P	Significant
Constant		109.250	1.190	91.81	0.000	
Days		-8.8375	0.4345	-20.34	0.000	
Days*Diets						
PLC	6.300	2.5375	0.7526	3.37	0.003	**
PEN	2.833	6.0042	0.7526	7.98	0.000	***
CLO	4.217	4.6208	0.7526	6.14	0.000	***
Control	17.675	-13.1625	0.7526	-17.49	0.000	***

Table 25a. Two-way ANOVA with day as a covariate on survival of *P.indicus* from M1 to PL stages on various feeding regimes. Control treatment was excluded in the calculations as it did not fit in the model (General linear model).

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Sigmificant
Diet	2	770.311	79.326	39.663	4.37	0.023	
Day	1	158.669	158.669	158.669	17.46	0.000	
Repl(Diet)	6	367.200	166.931	27.822	3.06	0.020	
Diet*Day	2	38.406	38.406	19.203	2.11	0.140	Ns
Repl*Day(Diet)	6	105.000	105.000	17.500	1.93	0.113	
Error	27	245.325	245.325	9.086			
Total	44	1684.91					

Table 25b. Comparison of larval survival rate (% day^{-1}) of *P. indicus* between M1 and PL stages.

Term	Mortality rate (% day^{-1})	Coeff	Stdev	t-value	P	Significant
Constant		93.750	2.269	41.32	0.000	
Days		-1.3278	0.3177	-4.18	0.000	
Days*Diets						
PLC	0.717	0.6111	0.4493	1.36	0.185	Ns
PEN	1.033	0.2944	0.4493	0.66	0.518	Ns
CLO	3.267	-0.9056	0.4493	-2.02	0.054	Ns

Table 26a. Two-way ANOVA with days as a covariate on growth of *P. indicus* larvae from PZ1 to PZ3/M1 stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Sigmificant
Diet	3	21.719	0.130	0.043	0.92	0.430	
Day	1	308.786	308.893	308.893	6554.06	0.000	
Repl(Diet)	8	0.790	0.305	0.038	0.81	0.595	
Diet*Day	3	6.094	6.122	2.041	43.30	0.000	***
Repl*Day(Diet)	8	0.790	0.790	0.099	2.10	0.034	
Error	668	31.483	31.483	0.047			
Total	691	369.662					

Table 26b. Comparison of growth rate (mm day^{-1}) of *P.indicus* from PZ1 to PZ3/M1 stages.

Term	Growth rate (mm day^{-1})	Coeff	Stdev	t-value	P	Significant
Constant		1.16632	0.01952	59.74	0.000	
Days		0.474959	0.005867	80.96	0.000	
Days*Diets						
PLC	0.415	-0.06005	0.01022	-5.87	0.000	***
PEN	0.455	-0.01947	0.01005	-1.94	0.053	Ns
CLO	0.440	-0.03479	0.01012	-3.44	0.001	***
Algae	0.589	0.11431	0.01025	11.15	0.000	***

Table 27a. Two-way ANOVA with days as covariate on growth of larvae on various diets fron M1 to PL stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Sigmificant
Diet	3	14.017	3.376	1.125	10.85	0.000	
Day	1	382.259	367.237	367.237	3539.72	0.000	
Repl(Diet)	8	1.001	1.228	0.154	1.48	0.161	
Diet*Day	3	1.376	1.444	0.481	4.64	0.003	***
Repl*Day(Diet)	8	1.076	1.076	0.134	1.30	0.242	
Error	707	73.350	73.350	0.104			
Total	730	473.079					

Table 27b. Comparison of larval growth rates (mm day^{-1}) of *P. indicus* between M1 and PL stages.

Term	Growth rate (mm day^{-1})	Coeff	Stdev	t-value	P	Significant
Constant		0.80952	0.06228	13.00	0.000	
Days		0.517013	0.008690	59.50	0.000	
Days*Diets						
PLC	0.518	0.00058	0.01487	0.04	0.969	Ns
PEN	0.527	0.00999	0.01455	0.69	0.493	Ns
CLO	0.559	0.04171	0.01462	2.85	0.004	**
Control	0.465	-0.05228	0.01611	-3.25	0.001	***

Table 28a. Survival data (%) for Figures 9a, b. Each value is a mean +/-s.d. (n=3).

Days	PLC	PEN	CLO	Control
1	100.00	100.00	100.00	100.00
2	100.00	100.00	100.00	100.00
3	99.50+/-0.87	100.00	100.00	99.00+/-1.73
4	92.17+/-4.19	97.83+/-2.93	98.17+/-2.36	82.33+/-5.03
5	83.67+/-2.52	94.5+/-2.78	93.00+/-5.41	59.83+/-5.69
6	81.00+/-2.65	91.67+/-1.76	87.67+/-6.51	33.17+/-4.04
7	79.83+/-3.18	90.67+/-2.75	87.63+/-5.89	16.5+/-6.54
8	79.67+/-1.26	89.17+/-1.89	85.33+/-9.75	11.5+/-2.59
9	78.83+/-1.61	89.00+/-3.04	82.17+/-8.31	10.50+/-4.71
10	77.83+/-3.55	87.67+/-2.32	79.17+/-7.01	9.3+/-2.93

Table 28b. Growth data (mm TL) fro Figures 10a, b. Each value is a mean +/- s.d. (n=3).

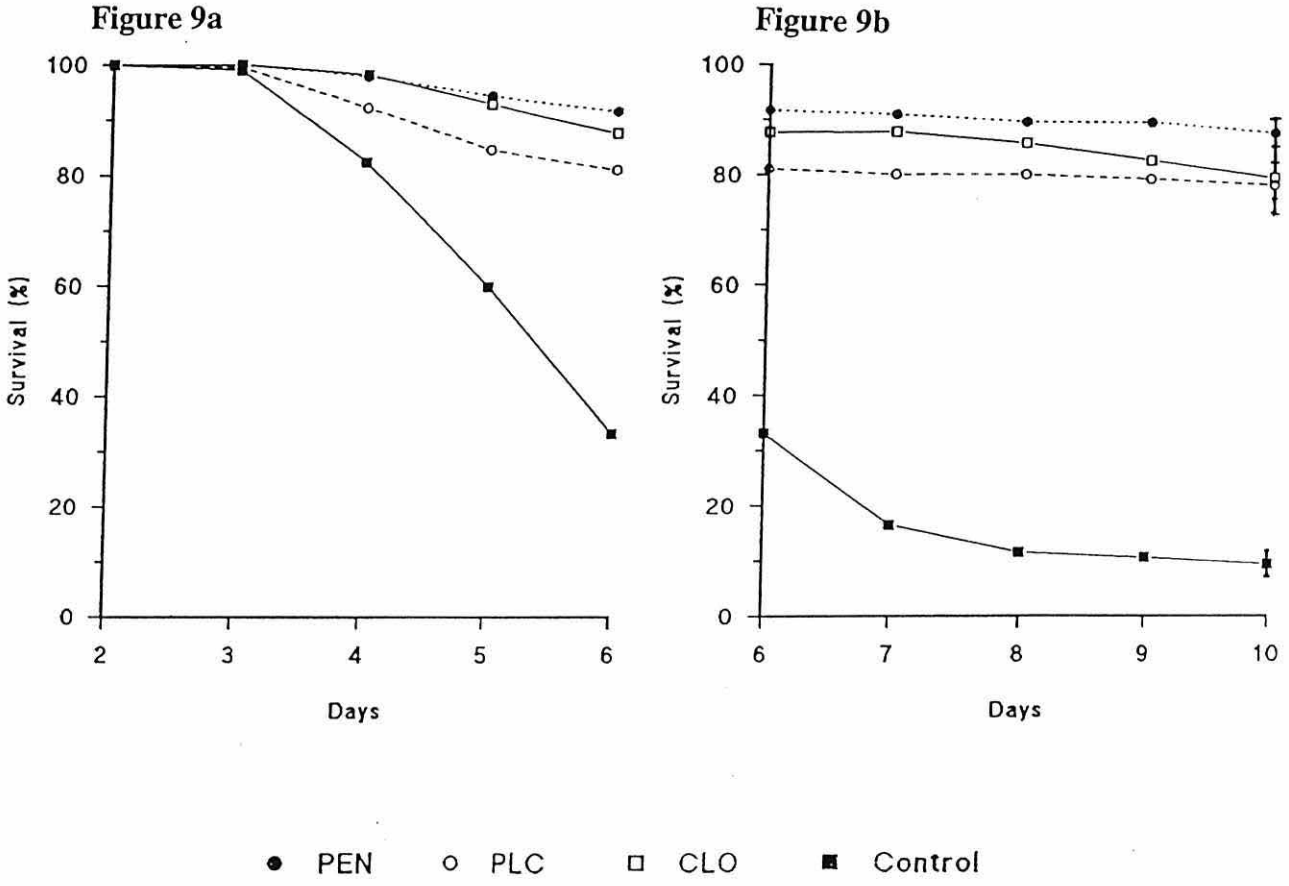
Days	PLC	PEN	CLO	Control
2	1.12+/-0.03	1.12+/-0.03	1.12+/-0.03	1.12+/-0.03
3	1.42+/-0.01	1.4+/-0.01	1.44+/-0.01	1.49+/-0.02
4	2.25+/-0.02	2.28+/-0.02	2.22+/-0.01	2.44+/-0.08
5	2.53+/-0.05	2.57+/-0.04	2.50+/-0.06	3.18+/-0.04
6	2.98+/-0.01	3.07+/-0.06	2.92+/-0.03	3.46+/-0.08
7	3.11+/-0.05	3.32+/-0.07	3.28+/-0.12	3.92+/-0.02
8	3.71+/-0.06	3.82+/-0.03	3.77+/-0.03	4.10+/-0.04
9	4.19+/-0.08	4.36+/-0.02	4.47+/-0.07	4.56+/-0.13
10	4.83+/-0.05	5.07+/-0.07	5.11+/-0.11	5.26+/-0.16

Table 29a. The nutritional profile of nematode diets fed in the first week of this experiment to *P. indicus* larvae from PZ1 to PL stages. PLC=placebo lipid-enriched, PEN=pigmented lipid-enriched, CLO=lipid-enriched nematodes.

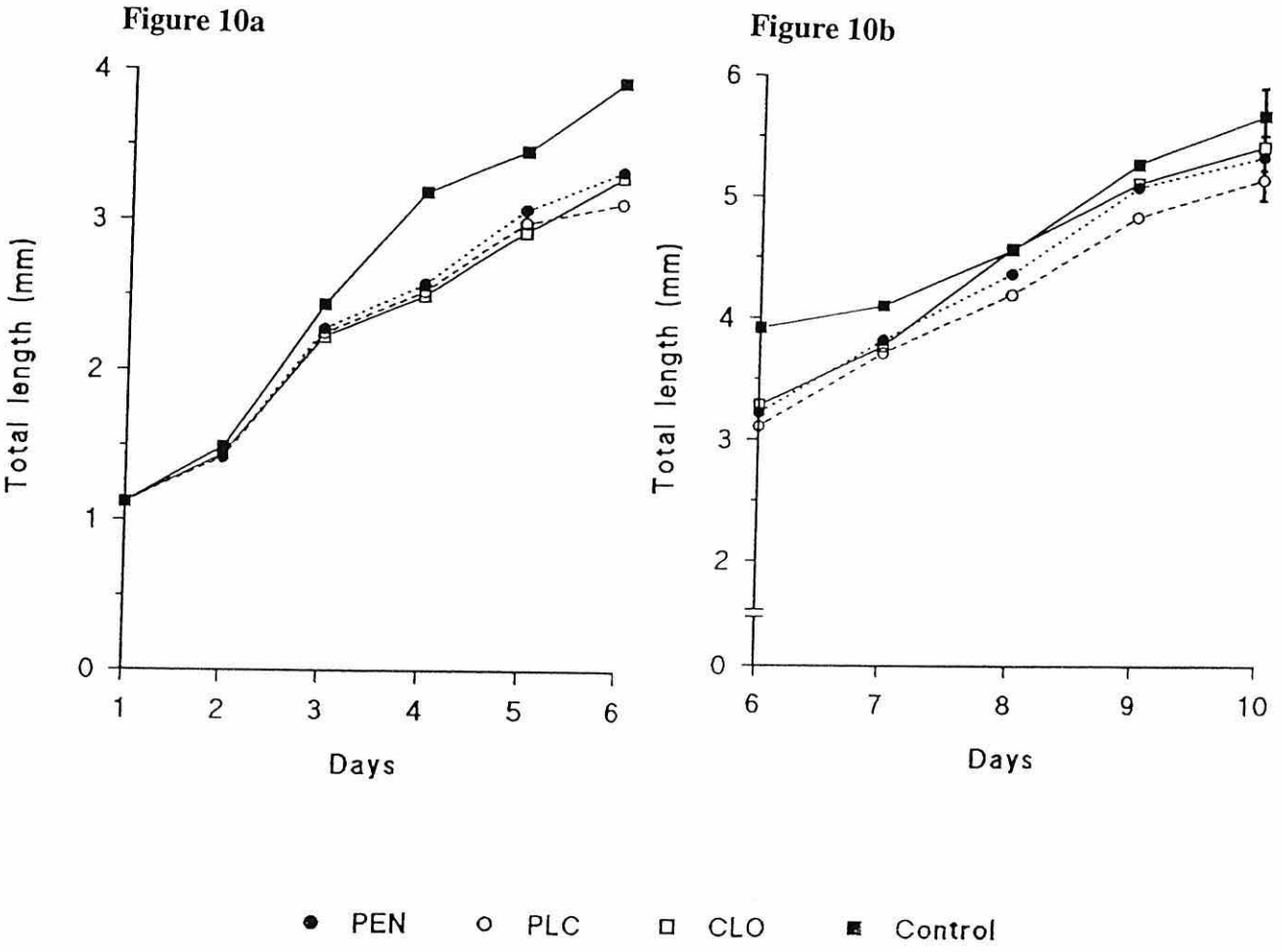
	PLC	PEN	CLO
Protein %	55.6	51.8	53.9
Lipid %	35.2	22.8	38.5
Fatty acids			
14:0	1.01	0.47	1.57
16:0	9.45	4.89	11.56
16:1ω-9	0.54	0.37	0.41
16:1ω-7	1.55	1.78	3.31
18:0	7.83	3.87	6.44
18:1ω-9	10.37	10.43	15.75
18:1ω-7	4.01	3.16	3.65
18:2ω-6	21.47	19.62	23.35
18:3ω-3	0.59	0.56	1.1
18:4ω-3	0.29	0.64	0.76
20:0	0.45	0.2	0.36
20:1ω-9	3.67	3.58	4.41
20:2ω-6	4.57	0.98	2.23
20:3ω-6	2.47	1.24	1.28
20:4ω-6	5.65	2.66	0.72
20:4ω-3	0.63	0.63	0.59
20:5ω-3	7.96	9.01	9.22
22:1ω-11	2.47	1.53	0.29
22:5ω-3	0.4	0.42	0.49
22:6ω-3	2.57	4.02	5.24
Saturates %	18.74	9.43	19.91
Monosaturates %	22.61	20.85	27.83
Polysaturates %	46.6	39.78	44.96
Sum %	87.95	70.06	92.72
ω-3	12.44	15.28	17.39
ω-6	34.16	24.50	27.58
ratio ω-6/ω-3	2.75	1.6	1.59

Table 29b. The nutritional profile of nematode diets fed in the second week of this experiment to *P. indicus* larvae from PZ1 to PL stages. PLC=placebo lipid-enriched, PEN=pigmented lipid-enriched, CLO=lipid-enriched nematodes.

	PLC	PEN	CLO
Protein %	59.6	54.9	54.0
Lipid %	34.6	32.0	31.9
Fatty acids			
14:0	1.5	1.13	1.75
16:0	9.56	6.92	9.47
16:1ω-9	0.24	0.3	0.21
16:1ω-7	3.15	2.97	3.55
18:0	3.32	3.38	3.18
18:1ω-9	19.48	16.1	18.65
18:1ω-7	2.93	3.39	3.55
18:2ω-6	28.81	26.32	25.5
18:3ω-3	0.99	0.85	0.97
18:4ω-3	0.82	0.98	0.99
20:0	0.36	0.29	0.28
20:1ω-9	3.85	4.21	4.68
20:2ω-6	0.49	0.7	0.45
20:3ω-6	0.64	1.88	0.6
20:4ω-6	1.91	2.07	1.64
20:4ω-3	0.34	0.59	0.46
20:5ω-3	4.49	7.59	6.04
22:1ω-11	2.4	2.4	2.86
22:5ω-3	0.51	0.59	0.59
22:6ω-3	3.22	4.1	4.24
Saturates %	14.74	11.72	14.68
Monosaturates %	32.05	29.37	33.5
Polysaturates %	42.22	44.67	41.48
Sum %	89.01	85.76	89.66
ω-3	10.37	14.7	13.29
ω-6	31.85	29.97	28.19
ratio ω-6/ω-3	3.07	2.04	2.12



Figures 9a and 9b. The survival (%) of *P. indicus* larvae reared on feeding regimes (9a) from PZ1 to PZ3/M1 and (9b) from M1 to PL stages. PEN=pigmented and lipid-enriched, PLC=placebo lipid enriched, CLO=cod liver lipid enriched, Control=mixed algae during protozoeal stages and plus *Artemia* during later stages. Results are a mean of 3 replicates.



Figures 10a and 10b. The survival (%) of *P. indicus* larvae reared on feeding regimes (10a) from PZ1 to PZ3/M1 and (10b) from M1 to PL stages. PEN=pigmented and lipid-enriched, PLC=placebo lipid enriched, CLO=cod liver lipid enriched, Control=mixed algae during protozoeal stages and plus *Artemia* during later stages. Results are a mean of 3 replicates.

DISCUSSION

Larval Development

The results obtained in experiment 1 demonstrate that a complete replacement of live algae and *Artemia* in the culture of *P. indicus* throughout all larval stages can be achieved using *P.redivivus* nematodes alone. The results presented in Table 5 show that the fastest larval development was achieved with the control feed followed by the combination of enriched nematode and algal co-feed (EN/algae), both during the protozoeal and later larval stages. This was demonstrated by the fact that at day 4 of the experiment larvae fed the control diet were at the M1 stage compared to 72% of the larvae fed EN/algae, the rest at PZ3 stage. However when the final survival at PL stage was reached at day 10 larvae fed EN/algae clearly supported a significantly higher larval survival at the 5% level compared to all other feeding regimes.

The results presented in Experiment 2 show that larval development is generally slower for single nematode feeds (non enriched nematodes (NEN), enriched nematodes (EN) and pigmented enriched nematodes (PEN)). This was demonstrated by the fact that larvae reared on all single nematode diets were at PZ3 compared to over 50% of larvae fed either nematodes / algal co-feeds or algae/*Artemia* control diet were at M1.

Alternative lipid sources were investigated in Experiment 3 and during PZ1 -PZ3/M1 the highest growth rate was achieved with EN nematodes (0.574 mm day^{-1}) and control diet (0.360 mm day^{-1}). Development from M1 to PL1 showed that Marilla oil enriched nematodes (MAR) fed larvae gave the highest growth rate (0.425 mm day^{-1}) compared to all the other treatments and control diets (0.360 m day^{-1}). The development of larvae in Experiment 4 fed on pigmented enriched nematodes (PEN) and cod liver oil enriched (CLO) nematodes metamorphosed into PL1 stage whilst placebo enriched nematodes (PLC) fed larvae reached this stage on day 10. Generally larvae fed control diets metamorphosed 1-2 days earlier.

In this study larval development and growth on live nematode diets was signficantly improved when a small amount of live algae was fed to *P. indicus* larvae for short periods together with the nematodes. Results presented in Figures 4a and 6a show that the algal co-feed promoted a higher

survival rate and better larval growth rate (0.831 - 0.927 mm day^{-1}) than mixed algae treatments during protozoeal stages. This agrees with Wilkenfeld (1984) who also observed that better growth, survival and larval development was obtained on nematodes with algae compared to nematodes alone. It was evident that nematodes fed with algal co-feeds promoted a better larval survival (38-63%) but a smaller total length (5.63-5.95 mm total length) at PL stages compared to algae/*Artemia* control treatment (32% and 7.49mm total length). However after mysis stages, growth rates were not significantly different between treatments with or without algae suggesting that an algal co-feed has positive effects during PZ1 -PZ3 but has no beneficial effects during M1- PL1.

Larval Growth

The highest larval growth rate from PZ1 -PZ3/M1 was achieved with a mixed algal diet with a total length of 3.98mm. The larval growth rate during the same period for larvae fed 30 cells of algae (*T. chuii/S. costatum*) for 24h with lipid enriched nematodes was significantly better than the larval growth rate during the same period than the larval growth rates of treatments fed enriched nematodes alone (0.62 mm day^{-1} and 0.51 mm day^{-1}) respectively. The difference in the colouration between the larvae fed pigmented nematodes and non pigmented nematodes could clearly be seen with the naked eye and microscopic examination as early as PZ1. Pigmented nematodes were not observed to improve the larval growth rate compared to non-pigmented nematode fed larvae. However this may have be caused by dirty cultures.

During M1-PL1 there appeared to be no positive effect to the larvae of lipid enrichment and pigment on larval growth. Table 4a and b demonstrates that larval growth and total length of larvae fed *Artemia* was significantly better than the larvae fed any of the other diets. The final total lengths at PL stages were significantly greater for larvae fed on algal co-feeds for a period of 24h at PZ1. This was significantly lower at the 5% level (0.30mm day^{-1}) compared to those larvae fed on other nematode diets during mysis stages.

It is apparent that the addition of algae for 24 h to the nematode treatment promotes growth of the larvae. It is known that algae appear to contain substances that trigger enzyme activity or enhance digestion and thus improve survival and growth on artificial diets (Amjad et al. 1993;

Jones et al. 1993; Rodrìguez et al. 1994).

In agreement with the results presented by the authors (Amjad et al. 1993; Jones et al. 1993; Rodrìguez et al. 1994), in experiment 1 the introduction of algae (15 - 30 cells μ^{-1}) with enriched nematodes (EN) promoted similar larval growth rates to those larvae fed live algal control treatments during protozoeal stages (Table 7). When larval growth rates were compared between day 1 and 4, larvae fed (enriched nematodes with algal co-feeds) EN/ algae diets supported the highest larval growth rate (0.93 mm day^{-1}). Larvae fed pigmented enriched nematodes (PEN) had higher growth rates than EN and NEN fed larvae (0.57mm day^{-1}) but were not equal to the EN/algae1 fed larvae. During development from M1 to PL stage larvae fed the algal/nematode diets showed a significantly lower growth rate compared to the control diet (0.60 mm day^{-1}). However the greatest larval total lengths were obtained from larvae fed the algae/ nematode regimes (7.49mm).

Experiment 3 explored the use of alternative lipid sources and during PZ1 -PZ3/M1 the highest growth rate was achieved with EN nematodes (0.574 mm day^{-1}) and control diet (0.360mm day^{-1}). Development from M1 to PL1 showed that Marilla oil enriched nematodes (MAR) gave the highest growth rate (0.425 mm day^{-1}) compared to all the other treatments and control diets (0.360 m day^{-1}).

Growth results obtained in experiment 4 show that larvae fed PEN nematodes gave a higher total length and growth rate (3.88mm and 0.455 mm day^{-1}) than placebo enriched nematodes (PLC) and cod liver oil enriched nematodes (CLO) fed larvae. Final total lengths were greatest for larvae fed the algal diet (3.92mm). During M1 and PL1 the highest growth rate was obtained for larvae fed CLO nematodes (0.599 mm day^{-1}). Larval growth rates for PEN and PLC fed larvae did not differ significantly at the 5% level during this period. The control fed larvae produced the lowest growth rate (0.465mm day^{-1}).

Larval Survival

Larval survivals shown in Figs 3 and 5 and Table 7 from PZ1 to PL stages fed all nematode feeding regimes except EN nematodes promoted significantly better larval survival rate and final survival than those of the control treatments. It was observed that nematodes with algal co feeds (EN/Alg1 and EN/Alg2) had a lower mortality rate compared to the control and EN treatments. The affect on survival of EN and NEN nematodes showed that EN treatments had the lowest final survival (10.33%) at PL and NEN had 51% final survival at PL and a lower mortality rate (4.14 % day $^{-1}$). 68% survival at metamorphosis was found with PEN nematodes, which had the lowest mortality rate.

Experiment 3 explored the use of alternative lipid sources. There seemed to be no affect on survival of larvae fed nematodes on alternative lipid sources apart from the fact that the highest mortality rate was found in larvae fed NEN nematodes (7.55 % day $^{-1}$) and that cod liver oil enriched nematodes CLO promoted the highest survival until M1 stage. However no significant differences were observed between treatments at the 5% level between PZ1 and M1 stages or M1 to PL1. Larval mortality decreased during mysis stages and the lowest survival was obtained with NEN and control diets.

The results obtained for survival in experiment 4 showed the effects of larvae fed either nematodes containing pigment or a placebo. The lowest mortality rates were achieved with PEN nematodes (2.83%). Highest mortality rates were found with larvae fed a mixture of algae during protozoeal stages (17.68%). At the M1 stage there was no significant difference between the treatments PEN, CLO and PLC. Survivals of 88%, 79% and 78% were obtained for PEN, CLO and PLC respectively compared to only 10.5% for the control. However these results were not linear and were therefore excluded from calculations.

Effects of an algal co-feed on larval development, growth and survival.

Numerous authors have shown (Table 30) that artificial diets fed alone generally promote comparable survival but slower growth in comparison to shrimp larvae fed on live diets (cited in Kumlu and Jones, 1995). In agreement with these authors the results presented in experiments 1, 2 and 3 show that survival is better than the control fed larvae.

Table 30 . Replacement of live feeds in commercial larval crustacean culture. (Summarised Table cited in Jones et al. 1993)

Species Penaeid Shrimp	Feed replacement	Result	Author
P. vannamei	Microcapsules + algae + Artemia 3-5 ml^{-1}	90% survival to PL5-7 (2 t tank)	Jones et al. (1987)
P. vannamei	Microcapsules + algae no Artemia.	80% survival to PL5-7 (25 t tank)	Jones et al. (1987)
P. stylirostris	Microcapsules + algae no Artemia.	65% survival to PL5-7 (25 t tank)	Jones et al. (1987)
P. monodon	Microcapsules only	3-29% survival to PL7 (1.2 t tank) settlement diet.	Jones et al. (1987)
P. monodon	Microcapsules + 10 cells μl^{-1} algae	76% survival, growth same as live fed control (2 L flasks)	Jones et al. (1987)
		90% survival, growth same as live feeds (lab scale)	
P. japonicus	microparticulate	Post-larvae fed enriched *Artemia* survive better than those fed non-enriched	Kanazawa (1985)
P. monodon	microparticulate diet + *Artemia*	*Artemia*	Tackaert et al. (1989)
P. vannamei			
P. japonicus		84% survival on 66% replacement algae	
		85% survival to mysis I	
P. vannamei	spray dried algae + *Artemia* microparticulate	Up to 62% survival to mysis I. Growth less than algae	Biedenbach et al. (1990)
P. monodon	microparticulate	42% survival to mysis I. Growth less than algae.	Galgani and Aquacop (1988)
P. indicus	microparticulate		Galgani and Aquacop (1988)
P. vannamei			Gagani and Aquacop (1988)

If improvements are to be made to the results obtained for growth, development and survival of larvae fed on alternative live and artificial diets, then a better understanding of larval digestive morphology, physiology and digestive enzymes of the commercially important penaeid shrimp species is required. It is known that the hepatopancreas (HP) is the main production site of digestive enzymes. Studies by Lovett and Felder, (1989, 1990a, 1990b) on *P. setiferus* larvae and Abubakr and Jones (1992) with *P. monodon* reveal that penaeid larvae possess anterior midgut diverticulae (AMD) which are the centre of enzyme release during protozoeal stages until the HP is fully developed. Survival of penaeid larvae on artifiial diets is attributed to the relatively high digestive enzyme activity present during early larval stages (Jones and Kurmaly, 1987).

The same authors also found that superior growth and survival was found using a mixed algal source during the herbivorous stages (*S.costatum* and *T. chuii*) compared to a single algal source for *P. indicus* larvae (Kumlu and Jones, 1993). Kumlu and Jones (1995) presented results that indicated that *P. indicus* larvae can be reared in the absence of an animal prey from PZ1 to PL1/2 with a 66% survival an a total length of 5.83mm on the MED plus 15 cells/μl^{-1} of frozen mixed algae. Research by the authors Le Vay et al.,(1993) show that tissue trypsin activity on live diets is low during PZ1 stage rising sharply during herbivorous stages (PZ1-PZ3) reaching a peak at M1 and falling during subsequent stages to the PL1 stage. Peak activities of all digestive enzymes found in penaeid larvae examined to date occur at late protozoeal and /or early mysis stages with low activities during early postlarval stages. Slower responses of digestive enzymes activity at this stage may be the reason why high mortalities occur when penaeid larvae are reared exclusively on artificial diets during early days of culture. Once the larvae pass this critical stage, their digestive capability appears to be better able to respond to the microencapsulated diet (MED) especially once the mysis stages are reached (Kumlu and Jones 1995).

Enzymatic digestion, as opposed to mechanical breakdown of food, is important in penaeid larvae as the foregut is devoid of masticatory ossicles and poorly developed, without a funtional gland filter for much of early life (Lovett and Felder 1989, 1990b, Abubaker and Jones 1992). Present results may also agree with the findings of the authors Kumlu and Jones (1995) as growth of larvae fed on nematode treatments alone compared to control diets and EN/algae regimes were significantly inferior. This may have been due to the inability of the larvae to actually digest the

cuticle of the nematodes during the early larval stages. However the addition of algae for the first 24 h as stated earlier, introduces substances that trigger enzyme activity to enhance digestion and thus improve survival and growth. The production of high levels of proteolytic enzymes for algal feeding, previously observed for penaeid larvae, may be a more general response amongst planktonic crustaceans feeding at the primary consumer level. In addition, there is now evidence, for penaeid protozoea, that microalgal compounds actually stimulate the production of trypsin like enzymes (Jones et al., 1993; Rodriguez et al., 1994) (cited in Kumlu and Jones, 1997).

In contrast to the situation in protozoeal stages, algae do not appear to provide enhancement of digestive enzyme activity in mysis *P. indicus* larvae (Kumlu and Jones, 1995). The results presented in experiment 3 show that from M1 to PL1 the highest growth rates were obtained for larvae fed on marilla and cod liver oil enriched nematodes and were significantly different from the controls and diets containing nematodes with an algal co-feed. There are two schools of thought on enzyme response to changes in dietary components. Harris and Paffenhofer (1976) and Hofer (1982) suggest that increased enzyme secretion is a response to shortage of a dietary substrate to maximise uptake of a scarce nutrient, whilst others (see Lovett and Felder 1990b) propose that enzyme secretion is intensified in response to the most abundant food substrates (cited in Jones et al., 1992).

Effect on larval development, growth and survival fed lipid enriched and non enriched nematodes.

Table 31 summarises available literature on nutrient requirements for penaeid larvae obtained by feeding known levels of nutrients on diets. The total dry weight percentages of lipid, protein and fatty acids of the nematodes (Tables 21a and 21b) were at the levels required by penaeid larvae. However little is known about the nutritional requirement of penaeid larvae (Kanazawa 1985, 1990). Kanazawa (1990) and Chen (1993) described levels of between 23-57%, 15-25% and 6-10% for levels of protein, carbohydrate and lipid respectively as nutritional requirements for penaeid larvae. The results shown in Tables 13, 21 and 22 reveal that *Panagrellus sp.* is similar to that of *Artemia,* has a similar level of protein and lipid to that of *Artemia* (Biedenbach et al., 1989, Le Vay et al., 1993). Kahan et al., (1980) also showed that the amino acid

Table 31. Nutrient requirements and dietary levels for penaeid larvae estimated from feed trials (cited in Jones et al. (1992)).

Species	Nutrient level	Comment	Author
PROTEIN			
P. japonicus	45-55%	Given 15-25% carbohydrate	Kanazawa (1989)
P. japonicus	43%	Better than 50%	Besbes (1987)
P. monodon	48-52%	Best growth on live diets	Kurmaly et al. (1989b)
P. monodon	51-56%	Best growth on encapsulated diets	Kurmaly et al. (1989)
P. monodon	30%	Microparticulate diet	Khanappa (1977)
P. monodon	23%	PZ1-M1 on ChaT. calcitrans	Tobias-Quinitio and Villegas (1982)
CARBOHYDATE			
P. japonicus	15-25%	Given 45-55% protein	Kanazawa (1990)
P. monodon	24-33%	Best growth on live diets	Kurmaly et al (1989b)
P. monodon	7.5%-18%	Best growth onencapsulated diets	Kurmaly et al. (1989b)
LIPID			
P. japonicus	1%	Dietary requirment for HUFA	Jones et al. (1979)
	3%	HUFA	Guillame (1990)
	1%	Phospholipid	Kanazawa (1990)
		Cholesterol	Teshima et al. (1983)
P. monodon	4.3%	Total lipid	
	3.4%	HUFA (Z1-3)	
(live feeds)	16%	Total lipids	
	12.4%	HUFA (M1-PL1)	
P. monodon	3.5%	Total lipids	
	26.1%	HUFA (PZ1-3)	
VITAMINS (M1-M3)			
P.japonicus		Carotene, thiamine, nicotinic acid, biotin, cyanocobalamin, riboflavin, pyroxidin, folic acid, vitamin D, choline, inositol, Ascorbic acid, vitamin E	Kanazawa (1986)

composition of *Panagrellus sp.* is similar to that of *Artemia*. Colvin (1976) stated that 43% protein was optimal for feeding *P. vannamei.* Biedenbach et al., (1989) reported that the protein and lipid levels of *P. redivivus* 48.3% and 17.3% respectively were therefore adequate for feeding *P. vannamei.*

It has been shown by (Léger and Sorgeloos, (1992) and Rees et al., (1994) that highly unsaturated fatty acids (HUFA) are important in the nutrition of penaeids. Marine shrimp have a poor ability to synthesise these fatty acids (Jones et al., 1979a; Kanazawa et al., 1979). A diet containing 1% of 22:6ω-3 promoted the highest survival, moult frequency and wet weight gain in *P. chinensis* juveniles (Xu et al., 1993). Tables 25 and 33 demonstrate that NEN nematodes containing ω-3 HUFA had only 0-0.5% of 22:6ω-3 and between 1.29 to 1.97% of 20:5ω-3. The results obtained in Experiments 1 and 2 show that too high a level of the HUFAs 20:5ω-3 and 22:6ω-3 may cause mortalities and abnormal larval development in agreement with Rees et al., (1994). Poor results were recorded with lipid enriched nematodes (EN) which gave high larval mortality rates (12% day^{-1}) and only 10% survival at metamorphosis. However pigmented lipid enriched nematodes (PEN) or EN/Alg 1,2,3 (enriched with the same lipid, capelin oil) gave low mortality rates.

The differences between the diets was that EN nematodes had double the level of EPA, whereas PEN nematodes had higher levels of lipid (33%) and DHA. Normal larval development was observed for larvae fed on a diet EN nematodes plus 30 cells μl^{-1} of live algae for only 24 h. It could be suggested that the imbalance of nutritional content of the EN nematodes is compensated by the addition of algae.

The results presented in Tables 21a and 21b show that the lipid levels can be manipulated with the addition of a lipid to the corn oil fraction of the nematode medium in agreement with Rouse et al., (1992). However the lipid enrichment of the nematode caused a decrease in total protein content from 74.4 -77.0% (NEN) to 52.9-64.6% (CLO) with a slight increase in lipid levels. The lipid enrichment increased for the ω-6 series of fatty acids from 2.21 - 2.94% (NEN) to 11.87%-12.26% (EN), 14.46-14.9%(MAR) and to 10.62- 11.28%(CLO). EPA also increased from 1.29%-1.42% (NEN) to 5.17-5.24% (EN), 6.0 -6.83 (MAR) and to 5.33 - 5.73%(CLO). The ω-6

series (DHA) was also improved from 0-0.8% (NEN) to 3.91-4.0% (EN), 3.74-4.39% (MAR) and to 2.79-2.86%.

Larvae fed CLO nematodes showed the lowest mortality rate (2.3% day^{-1}) during the postzoeal stages. Larvae fed on the EN and MAR nematodes also had significantly lower mortality rates (3.97-5.40 % day^{-1}) compared to the larvae fed on NEN nematodes (7.55% day^{-1}) and control live mixed algae (6.56% day^{-1}). However EN nematodes (capelin oil enriched) had a growth rate (0.574 mm day^{-1}) equal to that of larvae fed the control diet (0.576 mm day^{-1}) between PZ1 to PZ3/M1 stage. The results demonstrate that cod liver oil in the lipid source improves the survival where as capelin oil and Marilla oil favour growth. It is suggested that for future investigations a combination of these oils in the corn oil fraction of the nematode medium could be used to improve both growth and survival.

In experiment 3 the presence of high levels of 20:5ω-3 and 22:ω-3 may have attributed to a better larval performance. Xu et al., (1993) stated that the HUFA 20:4ω-6 (arachidonic) may play an important nutritional role for penaeids. However since a decrease existed in the levels of 20:4ω-6 in EN nematodes better larval survival and growth may have been caused by the presence of high levels of 20:5ω-3 and 22:6ω-3. *P. indicus* larvae has a limited capacity to elongate and desaturate linoleic and linolenic acids to C20 and C22 fatty acids and shows a similar fatty acid requirement to other penaeid species (Read 1981). These results do not show conclusively that lipid enriched nematodes enhanced larval growth and development. This may be due to the imbalance and lack of fatty acids in the nematodes during the larval culture (Rees et al., 1994).

The effect of pigmentation on larval development growth and survival

In this study the results of pigmentation were visible under a microscope after 24 h of feeding. Pigment could be seen in the tail and appendages of the larvae. However nematodes obtained from pigmented cultures were very dirty due to the difficulty of extracting nematodes from pigmented medium. This caused immediate larval fouling leading to high mortalities during larval culture. However larvae fed the pigmented nematodes at metamorphosis were distinctly pigmented compared to larvae fed non-pigmented (EN or NEN) nematodes.

Present results show that when algae (*Tchuii/S. costatum*) in low cell concentrations (15-30 cells μl^{-1}) were fed to *P. indicus* larvae for short periods such as 24 to 48h along with nematodes the larvae were better pigmented than larvae fed nematodes alone. However when the algal feeds ceased in experiment 1 and 2 the colour of the larvae gradually faded until metamorphosis. If well pigmented PLs are to be produced, algae should be provided to the larvae as a co-feed in addition to nematodes during all larval stages (Kumlu 1995). Results do not give any clear indication about any positive effects that the pigment (astaxanthin) has on the growth of *P. indicus* larvae in this study. The data in experiment 1 showed that the larvae fed PEN nematodes displayed a better larval growth rate than EN nematodes between PZ1 to PZ3/M1 stages. The larvae fed PNE nematodes had signficantly inferior larval growth rates to NEN nematodes during these stages (Table 1). These results may have been due to the difficulty in obtaining clean nematodes. Table 7 shows that in experiment 2 PEN nematodes promoted a better larval growth rate between PZ1 and PZ3/M1 (0.570mm day^{-1}) than EN or NEN nematodes. However results in experiment 4 also show that PEN nematodes promoted significantly higher larval growth rate (0.455 mm day^{-1}) than PLC nematodes or CLO nematodes (0.415-0.440 mm day^{-1}) during protozoeal stages. No significant difference was observed in the growth rate of the larvae fed PEN and PLC nematodes during mysis stages. Table 22 shows that the CLO nematode fed larvae displayed significantly better larval growth than those larvae fed PEN nematodes.

In agreement with Border et al., (1986) and Chien and Jeng, (1992) (cited in Thongrod et al. 1995) who reported that carotenoid supplementation increased the survival rate in kuruma shrimp, and there are indications that carotenoid deficiency reduces the growth rate in shrimp. Present results indicate that pigmented nematodes promote increased larval survival, but results suggest that pigment in nematodes fed to the larvae may not have any benefit on the growth during larval development of *P. indicus*. Thongrod et al., (1995) claimed increased survival was significant up to a dietary astaxanthin supplementation of 60 $mg.kg^{-1}$.

The conclusion from the present study is that pigmentation of nematodes delivered to *P. indicus* larvae significantly improves the larval survival compared to non pigmented nematodes. However there is no evidence to suggest the benefit of a pigment additive in the larval growth of *P. indicus* larvae. Under commercial conditions, feeding penaeid larvae on a combination of

algae and nematodes would produce a larval growth and survival equal if not better than an algae/*Artemia* feeding regime. It appears that the use of an algal co-feed will compensate for any possible nutrient deficiency such as vitamins and fatty acids (Rouse et al., 1992) and any imbalance in the nutritional content of pigmented and lipid enriched nematodes (Kumlu 1995).

Future work

In order to assess the commercial viability of nematode species such as *P. redivivus* as a food source for penaeid larvae it will obviously be necessary to examine the economics and feasibility of their mass culture. To achieve this aim it is essential to identify the following;

1) The earliest larval stage at which nematodes may be fed to penaeid larvae to achieve growth and survival which is at least equal to that achieved under standard feeding practices. The trials demonstrated that all the species of nematodes actually tested were unsuitable as a food source for *M. rosenbergii*. However the prelimnary trials with *P. indicus* demonstrated that the nematode *P. redivivus* was a possible alternative food source to *Artemia* for larval culture. It is believed that some of the smaller nematode species should be re examined as potential algal substitute for the early larval stages of *P. indicus.*

2) The optimum feeding density at each larval stage

3) The optimum level of lipid/vitamin enrichment required.

4) A complete nutrient profile of *P. redivivus.*

5) Combination of fish oils in the corn oil fraction of the nematode medium could be used to improve both growth and survival.

6) It would also be necessary to run laboratory trials with some of the more commercially important penaeids such as *P. monodon* and *P. japonicus.*

CHAPTER 7

PRELIMINARY FISH LARVAL TRIALS

Research for this chapter was jointly conducted by myself who cultured the nematodes and Mr. Andrew Barbour who conducted the fish larval trials at the Hunterston Fish Laboratories.

7. PRELIMINARY FISH LARVAL TRIALS

INTRODUCTION

Very few fish species have been successfully reared exclusively on artificial diets (Jones et al., 1993). However early stage larvae can be reared on artificial diets as a partial replacement to live feeds. This has been found with *Micropterus dolomiei* (Ehrlich et al., 1989), *Chanos chanos* (Marte and Duray, 1991), *Lates calcarifer* (Walford and Lam, 1991) and *Sciaenops ocellatus* (Holt 1991). Many marine larvae hatch with a small yolk reserve and therefore start to feed at an early stage (2-3mm) compared to fresh water fish larvae which are large at hatching (12-25mm for salmonids). Successful replacement for live feeds with marine larvae has been extremely limited (Jones et al., 1993). Complete weaning onto artificial diets can be achieved earlier than is currently practised for *Dicentrarchus labrax* (Person Le Ruyet, 1991) and potentially for *Solea solea* (Appelbaum 1985, cited in Jones et al., 1993). In most cases the mass rearing of marine fish larvae of commercial value depends on the abundance of a suitable live food supply. A suitable food must have the correct nutritional ingredients as well as proper physical and palatability properties to be accepted by the fish larvae. Freshly hatched *Artemia sp* nauplii meet these requirements and are digestible (Munilla - Moran and Stark, 1989 , cited in Jones, 1993). Although *Artemia* has many advantages as a larval food, they also have several disadvantages, including variations in hatching rate (Sorgeloos, 1980) and nutritional value (Johns et al., 1980; Sorgeloos, 1980; Watanabe et al., 1980) Another major disadvantage in the use of *Artemia* is their high cost, US$50-80/Kg (Radwin and Rouse, 1990). The size of food organisms to fit the small mouth cavity of marine larvae is of a major importance. The number of food organisms available for breeding marine fish larvae is small, however nematodes have been suggested for fresh water fish fry as a successful alternative live feed to *Artemia* or as an addition to algal feeding regimes (Bruun, 1949; Thomson, 1963, 1966; Sterba, 1967; Vallet et al., 1970; Hofsten, 1983; Kahan and Appel, 1975; Kahan, 1983 and Rottman 1991).

The objective of this chapter is to investigate the potential of *P. redivivus* and *C.elegans* as a larval feed replacement for marine fish larvae. Experiments 1 to 6 explored the use of nematodes

as a larval feed for *Scopthalmus maximus.* Initially the nematode *Caenorhabditis elegans* was assessed as a first feed for *S. maximus*, this was because of the small size of the nematode was approximately 500µm compared to *P. redivivus* which is approximately 1200 to 1500µm in length. Due to the negative result obtained in the first trial higher feeding rates were investigated. Day 20 larvae were also fed *C. elegans* larvae in an attempt to get the larvae to feed on the nematodes. It was then assumed that *C. elegans* was nutritionally inadequate to support the growth of the larvae and in experiment 4 *C. elegans* grown on lipid enriched medium were given to the fish larvae, these nematodes had elevated levels of 22:6ω-3 and 20:5ω-3.

Little success was obtained in the feeding of *C. elegans* to *S. maximus* larvae. As a result the suitability of *P. redivivus* as a food for *S. maximus* larvae was assessed (experiment 6). The palatability of *P. redivivus* was also investgated with *Pleuronectes platessa*, *Ctenolabrus rupestris* and *Clupea harengis* (experiments 7, 8 and 9). Data analysis was restricted to simple mean calculations on lengths and % survival counts due to the conclusive results obtained.

MATERIALS AND METHODS

Larval trials were performed in 5 litre round bottom glass flasks incubated in a water bath at 16^0C with a daily 1^0C increment from day 3 post hatched larvae. 100 day 3 post hatch larvae were placed in the vessels and fed at a rate of 5 nematodes ml^{-1} under axenic conditions. Control larvae were fed *Brachionus plicatilis* and *Monochrysis lutheri* freely. Experimental flasks were fed *C. elegans* and *M. lutheri*(experiments 1 to 7) and *P. redivivus* (experiments 8 and 9). Ten larvae were removed to measure dry weight and overall survival. Two replications for each treatment were made. Sea water was irradiated with UV light to reduce bacterial contamination and cartridge filtered through 0.2µm to eliminate food sources from the larvae. Optimum predator prey interaction and O_2 circulation was maintained without damage to the fish larvae by contiuous aeration supplied via silicone rubber tubing with glass tubes at the tip. Complete water exchanges were performed every 2 days when the larvae were also counted and a sample 20 were measued in length and staged. 50% water changes were made on alternate days. Live food counts were performed on individual flasks and appropriate amounts of the feed were added to maintain specific feeding regimes detailed below. Samples of the larvae were measured at the

commencement of each trial.

Nematode cultures

Live nematodes were soaked in small cubic sponges (10 million in 3g sponge in 10ml of water) and kept at 4^0C in an ordinary refrigerator. They were released in distilled water, concentrated and counted (three times) in a 1ml counting chamber before they were fed to the larvae. The size range of the nematodes was generally between 150µ and 900µ. Nematodes were produced in a monoxenic liquid medium with attempts to manipulate their nutritional profile to supply a more adequate food for penaeid larvae. Culture conditions of the nematodes were;

Bacteria : *Escherichia coli* (OP50)

Medium (w/w): 10% kidney, 1% yeast extract and 3.5% corn oil

Flasks : 250 ml baffled flasks with 50ml of medium,

Culture temperature : 22^0C.

Shaking incubator : 170 rpm.

An inoculum of 2000 nematodes ml^{-1} was added to the flasks which had been previously (24h) inoculated with *Escherichia coli.* The flasks were incubated at 22^0C for 10 -22 days in an orbital incubator, at approximately 150, 000 nematodes ml^{-1}. The nematodes were extracted from their medium by sedimentation and decantation in fresh water. They were packed in 15 x 18 cm high density polyethylene bags containing 2g of foam blocks and 10-20 ml of water. When large numbers of nematodes were required, they were produced in mass quantities in fermenters (10 litre or larger). These vessels were inoculated with 2000 nematodes ml^{-1} and harvested when the population reached a maximum of 174,000 nematodes ml^{-1} in 18-25 days. Generally, nematodes were regularly obtained from myself.

***Scopthalmus maximus* larvae fed *C. elegans* nematodes.**

Experiment 1

Scopthalmus maximus larvae were placed in 5 litre round bottom glass flasks and incubated in a water bath at 16^{0}C with a daily 1^{0}C increment from day 3 post hatched larvae. 100 day 3 post hatch larvae were placed in the vessels and fed at a rate of 5 nematodes ml^{-1}. Control larvae were fed *Brachionus plicatilis* and *Monochrysis lutheri.* Experimental flasks were fed *C. elegans* and *M. lutheri.* 10 larvae were removed to measure dry weight and overall survival. Two replications of this experiment were made.

Experiment 2

The design of the experiment was as described in experiment 1. 3 replicates were used for each treatment with a dose rate of 16 nematodes ml^{-1}.

Experiment 3

Larval trials were performed in 10 litre round bottomed flasks incubated in a water bath at 19^{0}C (+/- 5^{0}C). Thirty four day 20 larvae were placed in the vessels and fed at a rate of 10 nematodes ml^{-1}. The control treatments were fed 10 ml^{-1}*Artemia.*

Experiment 4

The experimental design was as described above using the nematode *C. elegans* enriched with Caspanthin in cod liver oil in freshwater for 2 h. *Artemia* controls were not set up. 40 larvae per flask were used.

Experiment 5

Thirty larvae were stocked in 10 litre round bottomed flasks and placed in a water bath at 19^{0}C

(+/- 1^{0}C). The target feeding level was 12 - 20 nematodes per ml. Two replicate for each treatment were used. 24 h after the first nematode feed the fish were examined for the presence of food in the gut.

***S. maximus* larvae fed *P. redivivus* nematodes.**

Experiment 6

Two larvae were placed in 10 litre round bottomed flasks in a water bath at 19^{0}C (+/-). 3/4 (20 days+) larvae were used. The larvae were starved 18 h previously. Three treatments were assessed; 1. larvae fed nematodes nematodes, 2. larvae fed the control *Artemia*, 3. Starved. Target feed levels : *P. redivivus* 10/ml/day and *Artemia* 0.4/ml/day. Water exchanges in the flasks was 30% per day. The larvae were fed over a 7 day period.

Pleuronectes platessa* larvae fed *P. redivivus

Experiment 7

Fifty larvae were placed into 5 litre round bottomed flasks at 19^{0}C in a water bath under non - sterile conditions. Nematodes were offered at 20/ml. The control flasks were fed *Brachionus plicatilis*.

***C. rupestris* larvae fed *P. redivivus* nematodes.**

Experiment 8

C. rupestris larvae were stocked at 10 larvae per litre in 10 litre round bottomed flasks and fed *Monochrysis lutheri* from day 1 post hatch and *P. redivivus* from day 2 in the experimental treatments. Controls were fed either *B. plicatilis* or *M. lutheri*. Flasks were held at 15^{0}C and culture conditions were non sterile.

Clupea harengis* fed *P. redivivus

Experiment 9

Twenty *C. harengus* larvae were stocked in 5 litre round bottomed flasks at 10^0C and kept in a 14:10 hour light:dark regime. The experimental fish were given *P. redivivus* from day 4 post hatch on a non target density 'window' of 6 - 15 nematodes per ml. All flasks contained *M. lutheri.* Rotifers were added to the controls on day 5 and used as a co - feed in experimental flasks from day 10 to avoid starvation. 3 larvae were removed on each day for sampling.

RESULTS

S. maximus* larvae fed *C. elegans

Experiment 1.

C. elegans did not support good growth or survival beyond day 6 for both experiments (Table 1a and 1b). Feeding on nematodes by the larvae was not observed and consequently no nematode debris was observed in the guts of the larvae. In contrast 100% of the larvae in the control treatment had evidence of food in their guts.

Table 1a.(Treatment 1) Percentage survival of and dry weight gain of *S. maximus* larvae fed *C elegans.*

	EXPERIMENTAL FLASKS		CONTROLS	
Day	Dry weight (µg)	% Survival	Dry weight (µg)	% Survival
3	33.52 +/- 2.60	100	33.92 +/- 2.60	100
6	32.10 +/- 4.21	95	51.25 +/- 6.07	100
9	29.87 +/- 2.46	72	102.09+/- 12.40	82
12	-	0	170.91+/- 36.21	82

Table 1b. (Treatment 2).

	EXPERIMENTAL FLASKS		CONTROLS	
Day	Dry weight (µg)	% Survival	Dry weight (µg)	% Survival
3	34.61+/- 1.96	100	34.16+/- 1.96	100
6	35.11 +/- 2.94	100	55.46 +/- 7.21	100
9	26.39 +/- 3.21	64	98.41 +/-11.48	82
12	-	0	161.37+/-17.28	78

Experiment 2

Table 2 shows that after 12 days larvae in the experimental flasks had 0% survival compared to 83 % for the controls. Larvae were not observed to feed upon the nematodes. It is necessary for first stage larvae of *S. maximus* to form a prey image. It is obvious that this prey image was not formed with the nematodes and the larvae were unable to feed upon the nematodes.

Table 2. Percentage mean survival and growth rate of *S. maximus* larvae fed a higher dose rate of nematodes.

	EXPERIMENTAL FLASKS		CONTROLS	
Day	Dry weight (µg)	% Survival	Dry weight (µg)	% Survival
3	31.20 +/- 3.26	100	31.20 +/- 3.26	100
6	28.42 +/-2.61	92	47.40 +/- 6.21	100
9	26.06 +/- 3.10	43	92.39 +/- 8.61	88
12	-	0	173.20 +/-14.48	83

Experiment 3

Four fish were sampled from each of the flasks fed nematodes. On day 2 larvae from 1 of the flasks tested showed the presence of 1 nematode in the gut. No fish sampled from flask 2 had any gut contents. *Artemia* fed larvae had the highest survival rate compared to starved and

nematode fed larvae. Survival was higher in the starved controls than in the nematode fed larvae suggesting that these nematodes had an adverse effect. The nematodes were cultured monoxenically with a bacterium which may have remained in the gut. Subsequently the nematode and bacterium had been eaten or remained in the surrounding environment. The results in Table 3 show conclusively the negative effects of nematodes fed larvae.

Table 3. Mean mortality rates of *S. maximus* larvae.

Mortality Rates (no.s of fish larvae)						
Day	Nematodes		*Artemia*		Starved	
1	5	2	0	1	0	0
2	22	10	2	2	3	5
3	0	3	5	2	10	6
4	0	1	0	0	4	8
5	1	5	1	0	3	4
Survival	6%	30%	76%	85%	41%	32%

Experiment 4

Two larvae examined on day 2 were observed to have 1 nematode in their guts, but subsequent fish larvae examined showed no gut contents. Table 4 demonstrates that starved larvae had a higher survival than nematode fed larvae.

Table 4. Mean mortality rates of *S. maximus* fed enriched nematodes.

Mortality rates				
Day	Nematode		Starved	
1	2	0	1	0
2	4	2	0	0
3	2	0	2	5
4	12	15	6	8
5	5	7	3	8
Survival	0%	11%	65%	38%

Experiment 5

Table 5 demonstrates that approximately 29% of the later stage larvae after 24 h had evidence of nematodes in their guts. This could be due to chance or to the fact that this stage larvae is larger, stronger and has a more developed buccal cavity. The larvae are better able in dealing with the nematodes.

Table 5. Survival of *S. maximus* larvae fed *P.redivivus.*

	Age of larvae at start (days)	% Larvae with nematodes in gut after 24 hours	% Survival after 6 days
Experiment 1	27	20%	45%
	32	22%	50%
Experiment 2	24	0%	0%
	29	29%	23%
Experiment 3	29	0%	10%
	31	100%	40%
	34	28%	45%
Mean		28.7%	30.4%

S. maximus larvae fed P. redivivus nematodes.

Experiment 6

It was demonstrated from experiment 5 that *S. maximus* larvae consumed nematodes. However when survival was compared to *Artemia* and starved larvae it was shown that nematode fed larvae were inferior even to starved larvae. Results from this experiment indicate that *P. redivivus* does not provide a suitable feed substitute for *Artemia.*

Table. 6. Mortality of *S. maximus* larvae fed *Panagrellus /Artemia/* Starved.

Day	*Panagrellus*		*Artemia*		Starved	
1	0	0	10.5	0	0	0
2	10.5	26.3	15.8	0	15.8	15.8
3	36.8	36.8	10.5	5.3	31.6	26.3
4	15.8	15.8	21	10.5	21	21
5	26.3	15.8	0	10.5	10.5	10.5
Survival%	10.6	5.3	42.2	73.7	21.1	26.4

Pleuronectes platessa* larvae fed *P. redivivus

Experiment 7

Nematodes were not observed in the guts of *P. platessa* larvae on sampling. To prevent the starvation of the larvae fed nematodes, the rotifer *B. plicatilis* was added freely on day 14 to experimental flasks. With reference to the result it can be seen that experimental mean dry weights are in the region of 25% to 48% (Table 7a) of the control dry weights suggesting that *P. platessa* larvae were not feeding on the nematodes.

Control fish were given *P. redivivus* from day 20 to day 30 in addition to the rotifers, but no feeding was observed. Ten fish were crash weaned onto the nematodes but did not feed. Metamorphosed fish were also offered *P. redivivus* but no uptake was observed. It was thought that newly hatched larvae were unable to form a prey image of the nematode and therefore were unable to recognise the nematode as a food source.

Table 7a. Mean dry weight and length of *P. platessa* larvae fed *P. redivivus* and *B. plicatilis*

	Experimental flasks		Control	
Day	Dry weight (µg)	Length (mm)	Dry weight (µg)	Length (mm)
5	106 +/- 5	7.1 +/- 0.3	106 +/- 5	7.1 +/- 0.3
7	133 +/- 32	7.3 +/- 0.3	139 +/- 26	7.0 +/- 0.8
11	114 +/- 18	7.6 +/- 0.2	109 +/- 37	7.2 +/- 0.5
14	48 +/- 4	6.9 +/- 0.1	97 +/- 23	7.0 +/- 0.2
17	34 +/- 11	7.0 +/- 0.5	137 +/- 47	8.0 +/-0.5

Table 7b. Mean percentage survival of *P. platessa* larvae.

Day	Experimental flasks	Control
5	100	100
11	41	100
17	28	100

C. rupestris larvae* fed *P. redivivus nematodes.

Experiment 8

Nematodes were not observed in the guts of the experimental fish larvae. A maximum of 25% of the control fish larvae showed rotifer remains in the gut. The survival of the experimental fish was 0% by day 10 (Table 8), survival was not estimated in the control flasks. Mean survival over 6 trials in the controls of the post hatch larvae to metamorphosis was 2.6%.

Table 8. Mean length (mm) of *C. rupestris* larvae fed *P. redivivus* and *B. plicatilis* or *M. lutheri*

Day	Experimental flasks Length (mm)	Controls Length (mm)
0	2.35 +/- 0.16	2.35 +/- 0.16
1	2.40 +/- 0.15	2.37 +/- 0.19
2	2.42 +/- 0.21	2.41 +/- 0.19
3	2.55 +/- 0.36	2.48 +/- 0.22
4	2.67 +/- 0.28	2.59 +/- 0.37
5	2.72 +/- 0.31	2.69 +/- 0.28
6	2.80 +/- 0.35	2.81 +/- 0.22
8	2.79 +/- 0.31	2.98 +/- 0.24
10	-	3.20 +/- 0.35

Clupea harengis* fed *P. redivivus

Experiment 9

Nematodes were not observed in the guts of the fish in experimental flasks on any of the days tested. The results indicate that the fish larvae were not feeding on the nematodes but relying on the background algal levels hence growth was reduced in comparison to the controls (Table 9). Again it was thought that the larvae were unable to use the nematode as a food source as it was unable to recognise it as a food source. Enriched and unenriched trials showed nematode fed fish survived 1 day longer (non - significant by 't' test) and had debris in their guts, but no nematodes were observed.

Table 9. Mean dry weight and length of *C. harengus* larvae fed *P. redivivus*.

Day	Control flasks		Experimental flasks	
	Dry weight(µg)	Length (mm)	Dry weight (µg)	Length (mm)
0	230 +/- 27	9.3 +/- 0.1	230 +/- 27	9.3 +/- 0.1
6	230 +/- 12	9.7 +/- 0.2	173 +/- 6	9.5 +/- 0.2
8	256 +/- 71	10.4 +/- 0.3	156 +/- 19	10.1 +/- 0.2
10	189 +/- 80	11.8 +/- 0.1	142 +/- 5	11.8 +/- 0.1
12	286 +/- 32	12.2 +/- 0.7	130 +/- 12	11.8 +/- 0.2
14	142 +/- 71	11.9 +/- 0.1	132 +/- 58	11.8 +/- 1.0
16	244 +/- 82	13.0 +/- 0.6	89 +/- 34	11.4 +/- 0.6
20	330 +/- 39	14.0 +/- 0.9	191 +/- 53	12.3 +/- 0.4
27	1039 +/- 596	17.2 +/- 2.0	505 +/- 102	14.9 +/- 0.8

DISCUSSION

C. elegans was not able to support the growth and survival of the first stage larvae of *S. maximus* in this study. In all of the experimental flasks larvae were not observed feeding upon the nematodes. No survival was observed in experimental flasks in experiment 1 compared to 82% survival observed in the controls. The effect of feeding the larvae with the nematodes was observed in the very low dry weight obtained for the larvae compared to the control treatments, approximately 26% of the actual dry weights of the controls. Beyond day 9 larvae did not survive on nematodes fed alone.

Increasing the nematode dose rate had no effect on the survival or dry weight of the larvae. Day 20 larvae had a survival rate lower than the starved larvae, indicating an adverse effect on the larvae. Three larvae were shown to have the presence of 1 nematode in their guts. The movement of the nematodes inside the larval gut may have a detrimental effect on the gut wall lining by causing a sloughing action due to the sinusoidal movement of the nematode. This may explain the low survival. The experiment was carried out in axenic conditions, however nematodes were cultured monoxenically with the bacterium *Escherichia coli*. This association of the bacterium with the nematode may have caused a detrimental effect to the larvae by ingestion or the presence of the bacterium in the environment.

The effect of enriching *C. elegans* with capsanthin in cod liver oil in freshwater for 2 h had no positive effect on the survival of the larvae. Survival was lower in the enriched nematode fed larvae compared to starved individuals. Two larvae examined however did show nematodes in the gut. Consumption of these nematodes may have been by accident. It was concluded that the large size of the nematodes was preventing the larvae from successfully feeding on them. As a consequence the larvae were not able to catch the nematodes or recognise them as potential prey.

The present study confirmed that *P. redivivus* and *C. elegans* are not suitable for the larval species of *S. maximus, C. rupestris, P. platessa* and *C. harengus* as total replacement feeds for algae and *Artemia.*

Enriched and non enriched *C. elegans* did not support growth and survival at any larval stages of *S. maximus* larvae. Larvae were not observed feeding upon the nematodes and evidence of feeding was not observed in the guts of the larvae. Post hatch larvae of all the species tested acquire a prey image after hatching and it was apparent that this was not formed on the nematodes. Nematodes were not considered as a possible food source, possibly due to the shape of the nematodes or the type of movement generated.

S. maximus larvae have an endogenous food supply for up to 3 days after hatching then the transfer to an exogenous food supply takes place, (Segner et al, 1993). At this stage a variety of enzymes are present. Although the digestive system is primitive in the turbot larvae, they should be able to digest the nematodes. It has been claimed that larvae rely on the digestive enzyme system of their prey (Govoni et al, 1986; Munilla - Moran and Stark, 1991). Digestion may be hindered by the presence of the chitin cuticle of the nematodes, it has been shown that the nematode cuticle remains undigested in the guts of some fish (Hofsten et al., 1983).

Older day 20 larvae were observed to feed on nematodes when higher dose feed rates were used. Further trials indicated that even though larvae attempted to feed their survival and growth was inferior to the starved larvae.

The trials with all species of fish larvae show that the nematodes *C. elegans* and *P. redivivus* can not be used as an alternative feed for these species because: 1) larvae were unable to recognise nematodes as a food source and unable to form a prey image, 2) On the occasion that the nematodes were ingested (accidently) the sinusoidal movement of the nematodes sloughed the gut lining causing serious damage to the larvae. 3) overall shape of the nematodes makes it difficult for the larvae to catch compared to *Artemia* which is more box like, 4) the presence of a growth bacterium with the nematode may have a harmful effect on the larvae.

Very little work has been previously published on the feeding of nematodes to marine fish larvae and varied results have been obtained with feeding nematodes to fresh water larvae. This is due to the density of fry used, temperature at which fry are raised and the regularity of the water changes, all these factors greatly affects growth (Kahan et al., 1981).

Rottman (1991) compared several live feeds with artificial diets for feeding grass carp (*Ctenopharyngodon idella*) and bighead carp (*Hypopthalmichthys nobilis*) larvae. It was concluded that the final mean length and dry weight of grass carp larvae fed *Panagrellus sp* was lower than the live feeds, but superior to the commercial diets tested. However for the bighead carp no significant difference was observed in the final length of the fish larvae fed *Panagrellus sp* or *Artemia.* Survival of grass carp and bighead carp larvae fed nematodes was excellent and exceeded 95% in all feeding trials.

Kahan et al (1983) showed that nematodes were satisfactory as a sole source of food for common carp (*Cyprinus carpio)* and silver carp (*Hypopthalmichthys molitrix*) larvae. However when nematodes were added to dry feed, growth was 1.39 - 1.84 times better, with higher survival than on each diet without addition of nematodes. The improvement to growth of the larvae was not due to the amino acid content which was similar to various other feeds (Table 10).

Rottman (1991) suggested utilizing a feeding program combining live food items with dry diet. This would be superior because; 1) they would meet more completely nutritional requirement of larvae for maximum growth and survival 2) provide a variety of food items to ensure consistent results 3) provide back up food supplies in case of production failures 4) it would probably be a less traumatic transition from live food for fingerling production by introducing the fish to an artificial diet early in life.

Table 10. Amino acid composition of *Panagrellus sp.* nematodes and some other food sources used in the rearing of fish fry, as weight percent of total amino acids. After Kahan et al ., (1983).

amino acid	*Panagrellus sp.*	*Cyclops spp.*	*Artemia spp.*	Alkan yeast	Diet KBF_2
Lysine	7.9 +/- 0.6	7.61	8.5	7.5	6.8
Histidine	2.9 +/- 0.2	2.22	2.3	2.3	3.2
Arginine	6.6 +/- 0.4	8.06	7.1	5.2	6.1
Aspartic acid	11.2 +/- 0.3	-	10.5	9.0	-
Threonine	4.7 +/- 0.8	4.86	4.8	4.9	4.1
Serine	3.7 +/- 0.9	-	6.4	4.5	-
Glutaminc acid	12.8+/1.7	-	13.6	12.7	-
Proline	5.4+/-0.2	-	5.9	4.1	-
Glycine	6.4 +/- 0.3	-	4.8	4.9	-
Alanine	8.8 +/- 0.1	-	5.5	7.4	-
Cysteine	0.0	1.41	-	1.1	1.4
Valine	6.4+/- 0.2	5.71	5.2	5.7	6.6
Methionine	2.2 +/- 0.6	0.45	2.3	1.8	1.4
Isoleucine	5.1 +/- 0.2	4.92	4.4	4.8	4.1
Leucine	7.7 +/- 0.1	11.68	7.6	7.2	9.0
Tyrosine	3.2 +/- 0.5	1.52	4.4	3.4	3.2
Phenylalanine	4.9 +/- 0.2	4.30	4.2	4.4	5.4
Tryptophan	2	0.78	-	1.3	1.1

Future work

It has been shown from the results that marine fin fish larvae do not utilise *C. elegans* or *P. redivivus* as a source of food. It was concluded that the physical size of the nematodes made it impossible for the larvae to utilise the nematodes. However results from the above authors with freshwater larvae and marine tropicals should not be dismissed. Further investigations should be undertaken to explore smaller nematode species or the use of *P. redivivus* as a feed in the culture of marine tropical fish species.

GENERAL CONCLUSIONS

8. GENERAL CONCLUSIONS

Of all the nematode species tested *Panagrellus redivivus* showed the most potential as a live food replacement for penaeid larvae. This is due to it's ease of culture and rapid propagation rates. The optimal growth of this nematode was dependant on initial inoculum level, culture temperature and medium composition.

It was established that 2000 nematodes per ml is required for the initial inoculum level in the culture. Growth of the nematode is optimum at 22^0C in a medium containing kidney as the nitrogen source. It is necessary to find a cheap source of nitrogen for use on a large scale culture. Chicken liver and bovril proved to be successful in supporting the growth of the nematodes. Bovril was the best choice as it is cheaper and easier to use. The use of chicken liver is not feasible on a large scale as this requires the presence of an enzyme or the use of a sieve to break down the sinew in the medium. The combination of bovril and yeast extract at certain concentrations proved to be detrimental to the growth of the nematodes. However the best combination of solid medium components was 2% bovril, 1% egg and 1% yeast extract and 3.5% corn oil.

After having established the optimum growth conditions for *P. redivivus* it was necessary to measure the relative amounts of proteins and lipids of the nutritional profile of the nematode to compare it to the nutritional profile of *Artemia*. On a dry weight basis of protein 40-50% and lipids 15-20% with polyunsaturated fatty acids (20:5ω-3 and 22:6ω-3) being 1% of the total lipid, the nematode *P. redivivus* compares to *Artemia* very well. With reference to the lipid analysis of *S. feltiae* and *H. megidis* and *Phasmarhabditis,* these nematodes have a lipid and protein profile within the desired selection criteria as described. The fatty acids were predominantly saturated. Fatty acids are important in providing energy reserves in infective juveniles, saturated fatty acids provided more energy than non saturated fatty acids (Stryer, 1988)

The essential fatty acids are ω-3 highly unsaturated fatty acids (ω-3 HUFA) such as eicosapentaenoic acid (EPA) 20:5ω-3 and docosahexaenoic (DHA) 22:6ω-3 is essential for normal growth of marine fishes (Watanabe, 1993), shrimps required the recommended levels of

6-7.5% in their feed.

It was shown that docosahexaenoic acid (22:6ω-3) is very low or absent in all species of nematodes. However eicosapentanoic acid (20:5ω-3) is present at reasonable level and is high in *P. hermaphrodita* and *P. redivivus*. When the corn oil component of the medium was analysed it was found to be devoid of the fatty acids 22:6ω-3 and 20:5ω-3. This suggests that these nematodes to a certain extent can manufacture these fatty acids and *S. feltiae* and *H. megidis* can manufacture 20:5ω-3. The levels of EFAs 22:6ω-3 and 20:5ω-3 can be successfully increased in the nematodes *P. redivivus, S. feltiae* and *H. megidis* when grown on a medium containing increased levels of capelin oil. This oil contains very high levels of EFAS. The presence of these fatty acids in these nematodes was well in excess of the dry weight of 1.5%. The nematodes very much reflect the medium composition in which they are grown.

The total analysis of *P. redivivus* reveals that the protein dry weight composition to be in the region of 45-50% and the lipid content to be in the region of 15-20%. *P. redivivus* contains high proportions of 18:2ω-6 and 18:1ω-9. The results also show that nematodes can grow on a variety of fish oils that possess high percentage of the EFAs 22:6ω-3 and 20:5ω-3.

The need for an inert carrier material was required for the storage and transport of nematodes to fish and shrimp hatcheries. Initial storage experiments with clay, vermiculite and peat produced very discouraging results. The use of polyacrylamide gels was avoided as this required the use of toxic chemicals which would not be beneficial to the nematodes. The use of high density polyethylene foam chips and alginate gel was investigated for their storage properties and ease of extraction of *P. redivivus*. The initial high temperature storage trials in foam chips reveals optimum numbers of storage in bags, water content and weights of foam chip to nematode paste. establishing the requirements for long term cold storage. During long term cold storage lipid and protein analysis was undertaken on the nematodes. These results revealed that nematodes could be stored for up to 4 weeks under these conditions at 5^0C in foam chips. Chemical analysis of nematodes showed an initial decline in total fatty acids for up to 10 weeks of storage. It was concluded that the major energy store of the nematodes was (18:1ω-9, 18:2ω-6, 18:3ω-3 and 18:4ω-3).

Further to improving the storage quality of the nematodes alginate beads were investigated. The increase in survival for long periods of time compared to foam chips was attributed to the prevention of movement of the nematodes within the alginate beads and therefore the decrease in energy expenditure. By chilling the reagents and nematode paste prior to encapsulation, this increased the numbers of nematodes that could be incorporated into each bead. Good survival was observed for beads containing 6000, 4000 and 2000 nematodes. Above these quantities irregularities in bead formation were observed for high concentrations and it was thought this may effect the accuracy of counts of nematodes obtained from the beads. It was established that survival of nematodes was superior in alginate beads compared to foam chips.

Initial trials of feeding *P. redivivus* to shrimp showed that although the larvae are healthy and active, they appeared pale compare to similar staged larvae fed *Artemia.* This was due to the canthaxanthin pigment in the diet. Nematodes were therefore pigmented with astaxanthin to increase the level of pigment presented to the shrimp larvae. Astaxanthin was used as it is the main pigment associated with shrimp and is the pigment more easily utilised by the shrimp. Astaxanthin incorporated into the medium of the nematodes did not affect the growth in any way. By increasing the concentration of astaxanthin in the diet above 1.5%, it did not increase the pigmentation of the nematodes. This was due to the fact that a 2% concentration of carophyll pink saturates the kidney medium and therefore pigment remains undissolved and can be seen in the medium. However if nematodes are harvested and stored in foam chips they lose their pigmentation rapidly over a period of days, this was due to the fact that nematodes accumulated the pigment in their guts.

Further investigation of storage experiments comparing pigment loss in foam chips and alginate beads revealed that pigment was maintained in the guts of nematodes for longer when stored in alginates compared to those stored in foam chips. 60-70% of nematodes contained pigment in their guts compared to no nematodes in foam chips after 12 days at 22^0C. This was attributed to the restricted movement of the nematodes within the alginate beads and hence the retention of pigment for longer periods of time in the gut. These results indicate that the optimum storage material for the nematode *P. redivivus* would be alginate gel beads stored at low temperatures between 0-5^0C containing 2000 to 6000 nematodes per bead.

The use of nematodes as a larval feed replacement proved unsuccessful for all species of fish larvae tested. Larvae were observed to feed upon the nematodes. Fish larvae used in the treatments appeared starved and the feeding of nematodes had no effect on the survival and the dry weight of the nematodes.

Evidence was observed for the feeding of *S. maximus* larvae on nematodes. Lipid enriched and non enriched *C. elegans* did not support growth and survival at any stage of *S. maximus* growth. It was concluded that unsuccessful use of nematodes was due to the inability of the fish larvae to form a prey image of the nematodes, therefore they were not considered as a possible food source. This may have been due to the shape of the nematodes and the type of movement generated. The trials were undertaken in axenic conditions, however nematodes were cultured in a monoxenic environment with the bacterium *E. coli.* The presence of this bacterium in the gut and on the surface of the nematodes could easily have been consumed by the larvae and released into the environment making conditions unfavourable for the growth and survival of the larvae. Thus producing larvae with survival and growth markedly inferior to the starved larvae. In 1 or 2 of the *S. maximus* larvae the *P. redivivus* was observed in the gut. The activity of these nematodes in the gut of young larvae appears to cause severe damage to the lining of the gut.

These trials indicate that the growth of larvae was not supported by the nematodes *C. elegans* and *P. redivivus*. It was concluded that: 1) larvae were are unable to recognise nematodes as a food source and are unable to form a prey image 2) On the occasion that nematodes were ingested (accidently) the rapid sinusiodal movement of the nematodes sloughed the guts of the larvae and caused severe damage to the larvae, 3) Overall shape of the nematodes makes it difficult for the larvae to catch compared to *Artemia* which is more box like in appearance, 4) The presence of a growth bacterium with the nematode may have a harmful affect on the larvae.

Positive results were obtained with *P. redivivus* fed to *P. indicus*. The study confirmed that *P.redivivus* can be used as total algal replacement feed at all *P. indicus* larval stages. In particular pigmented nematodes presented the most suitable alternative to the standard penaeid larval diet.

No advantage was observed in enriching these nematodes with high levels of 20:5ω-3 and 22:6ω-3 fatty acids. The fatty acid level of all the nematode treatments ie. NEN, PEN and EN was adequate to meet the lipid requirement of the larvae. It appears that by adding algae provides the early zoeal stages with a nutrient factor not available in the nematodes and was required during this early period to ensure growth.

Most of the nematode fed treatments gave reasonable survival and slower growth rates. This may have been an indication of inappropriate feeding rates. Nematodes settle out of suspension easily and that single feeds probably resulted in larvae being deprived of food for considerable periods of time.

PEN nematodes may have improved the visibility of the nematodes and subsequently enhanced capture and growth and survival. PEN and NEN fed larvae were similar throughout the trial, although the former exhibited superior survival rates and the NEN larvae actually moulted more slowly. Shrimp larvae also appeared pigmented compared to treatments fed unpigmented nematodes.

Future work

It would be necessary to repeat the fish larval trials by increasing the dose rate fed to the fish larvae this may increase the numbers of nematodes in contact with the larvae. The inadequate survival of these fish larvae may have been due to the nematodes falling out of suspension. Trials should also be undertaken with other species of penaeids to ascertain if this nematode would be a suitable feed for other species. Further work may also include the incorporation of vitamins into the medium ie. vitamin C. Improvements also should be made to the storage of these nematodes. Although the use of alginates improves the cold temperature storage of the nematodes, the packing density of nematodes to beads is not enough to supply the aquaculture industry with the quantities it demands. Also the ability to store this nematode at ambient temperatures would also be an advantage. It would also be an advantage to research other nematode species that would provide an alternative feed or an enhanced shelf life.

BIBLIOGRAPHY

Abubakr, B. and Jones, D.A. 1992. Functional morphology and ultrastructure of the anterior midgut diverticulae of *Penaeus monodon* (Fabricus, 1789) larvae. *Crustaceana*, **62** (2): 142-158.

Akhurst R.J., 1980. Morphological and functional dimorphism in *Xenorhabdus sps.*, the bacteria symbiotically associated with the insect pathogenic nematodes, *Neoaplectana* and *Heterorhabditis*. *J. Gen. Microbiol.* **121**, 303-309.

Akiyama D.M., Dominy W.G. and Lawrence A.L. 1992. penaeid shrimp nutrition. in: Fast, A.W and Lester, L.J. (Eds.), Marine Shrimp Culture: Principles and Practices, Elsevier. PP. 535-568.

Appelbaum, S. 1985. Rearing of the dover Sole, *Solea solea* (L), through its larval stages using artificial diets. *Aquaculture* **49**:209-221.

Amjad, S., D.A. Jones and K. Chitravedivelu. 1992. Advances in penaeid larval feed technology. Pages 29-45 in S.H. Cheah and T.HJ. Saidin, editors. Proceedings of seminar on new technologies in aquaculture. Malaysian Fisheries Society, Serdang Selangor, Malaysia.

Anderson B.A., Christie W.W. and Holman R.T., 1975. Mass spectrometric determination of positions of double bonds in polyunsaturated fatty acid pyrrolides. *Lipids* **10**:215-219.

Anderson R.V., 1966. Observations on the nervous system of *Aporcelaimus amphidysis* sp. (nematoda:Dorylaimoidea). *Can.J.Zool.* **44**: 815-820 (32).

Andrews J.W., Sick L.V. and Baptist G.J., 1972. The influence of dietary protein and energy levels on growth and survival of penaeid shrimp. *Aquaculture* **1**:341-347.

Aquacop. 1983. Penaeid larval rearing in the centre Oceanologique du Pacifique. In: Mcvey, J.P. (Ed.), CRC Handbook of Mariculture, Vol. 1, CRC press, Boca Raton, FL, pp. 123-127.

Balakanich S. and Samoiloff M.R., 1974. Development of nematodes behaviour : sex attraction among different strains of *Panagrellus redivivus. Can J. Zool.* **B**:835-845.

Barrett J., Ward C.W. and Fairbairn D., 197. Lipid metabolism in the free living nematodes *Panagrellus redivivus* and *Turbatrix aceti*. **38B**:279-284.

Barret J., 1988-1989. Drying of insect parasitic nematodes. Dept of Biological Sciences. University College of Wales Aberystwyth.

Barret J. and Butterworth P.E., 1984. Acetaldehyde formation by mitochondria from the free-living nematode *Panagrellus redivivus. Biochem. J.* **221**:535-540.

Beames C.G. and Fisher F.M., 1964. A study on the neutral lipids and phospholids of the Acanthocephala; *Macracanthorhynchus lirudinaceius* and *Moniliformes dubius*. *Comp.Biochem.Physiol.* **13**:410-412.

Bedding R.A., 1976. New methods increase the feasibility of using *Neoaplectana sps.* (Nematoda) for the control of the insect pests. *Proc. 1st Inst. Cell Invertebrate Pathology, Kingston, Canada.* 250-254.

Bedding R.A., 1981. Low cost invitro mass production of *Neoaplectana* and *Heteorhabditis* species (Nematoda). For the control of insect pests. *Nematol.* **27**:109-114.

Bedding R.A., 1984. Large scale production and transport of the insect parasitic nematodes, *Neoaplectana sp.* and *Heterorhabditis sp. Ann appl Biol.* **104**:117-120.

Bedding R.A., 1988. Storage of entomogenous nematodes. Int.Patent. WO88/O8668.

Bendich A., 1989. Carotenoids and the immune response. Symposium on the biological actions of carotenoids, 72nd annual meeting of The Federation of American Societies for Experimental Biology, Las Vegas May 2, 1988. pp.112-115.

Berntzen A.K., 1965. Comparative growth and development of *Trichenella spiralis* in vitro and in vivo with a redescription of the life cycle. *Exp. Parasitology.* **16**:74-106.

Biendenbach J.M., Smith L.L., Tamara K., Thomsen and Addison L. Lawrence., 1989. Use of the nematode Panagrellus redivivus as an Artemia replacement ina larval Penaeid diet. *World Aqaucult. Soc. No.2* **20**:61-71.

Biotechnology Australia, Nematode cream containing adsorbent and antimicrobial agent stable and prolonged storage and transport under anaereobic conditions. Int. Patent 85/03412, 1985.

Bird A.F., 1971. The Structure of Nematodes. New York:Academic Press.

Bird J.N. and Savage G.P., 1990. Carotenoid pigmentation in aquaculture. *Proc. Nutr.Soc. NZ* **15**: 45-56.

Bledsoe L.J., Shleser R.A. and Glude J.B., 1978. Development of research priorities for aquacultrure planning. Washington Sea Grant, WSG-MR, 78-2., University of Washington, Seattle, U.S.A. pp52.

Bollinger J.A. and Willet J.D., 1978. A method of age synchrony of *Panagrellus redivivus* in xenic culture. *Nematol.* **24**:398-403.

Brenner, S. 1974. *Genetics.* **77** pp.71

Brewster, L.F.S. 1987. The utilisation of artificial compound diets by larvae of the lobster, *Homarus gammarus.* M.Sc. thesis. University of Wales, Bangor, UK. 79pp.

Briggs M.P., 1946. Culture methods for free living soil nematodes. Thesis Stanford University California.

Britton, G., Armitt, G.M., Lau, S.Y., Patel, M., and Shone A.K. (1982), " Carotenoid Chemistry and Biochemistry" (G. Britton & T.W. Goodwin, eds.), Permagon Press, Oxford, pp. 237-251.

Bruun A., 1949. The use of nematodes as food for larval fish. *Nematol.* 69-99

Buecher, E.J., Hansen, E. L. and Yarwood, E.A. 1966. Ficoll activation of a protein essential for maturation of the free living nematode *Caenorhabditis briggsae. Proc. Exp. Biol. Med.* 121: 390-393.

Buecher E. J. and Hansen E. L., 1971. Mass culture of nematodes using continuous aeration. *J. Nematol.* **3**:199-200.

Buecher E.J. and Popiel I., 1989. Liquid culture of the entomogenous nematode *Steinernema feltiae* with it's bacterial symbiont. *J. Nematol.* **21(4)**:500-504.

Buecher E.J., Hansen E.L. and Myers R.F., 1970. Continuous axenic culture of *Aphelenchoides sp. J.Nematol.* **2**:189-190.

Burton G.W., 1989. Antioxidant action of carotenoids. Symposium on the biological action of carotenoids 72nd annual meeting of the federation of American Societies for Experimental Biology, Las Vegas May 2 1988. pp. 109-111.

Butterworth P.E. and Barret J., 1985. Anaerobic metabolism in the free-living nematode *Panagrellus redivivus. Physiol. Zool.* **58 (1)**:9-17.

Byerly L., Cassada R.C. and Russell R.L., 1976. The life cycle of the nematode *Caenorhabditis elegans*; wild type growth and reproduction. *Dev. Bio.* **51**:23-33.

Castell J.D., Sinnhuber R. O., Wales J.H. and Lee J.D., 1972. Essential fatty acids in the diet of rainbow trout (*salmo gairdneri*): Physiological symptoms of EFA defiency. *J.Nutr.* **102**, 87 -92.

Cavier, R. 1951. L'equipment enzymatique du liquide coelomique de *l'ascaris lumbricoides* Linne' 1758. C.R. *Acad. Sci. Paris.* **237**: 99-101.

Cavier, R. and Savel, J. 1953. Sur la presence d'une phoshorylase chez l'ascaris du porc, *Ascaris lumbricoides*, Linne' 1958. *C.R. Acad. Sci. Paris.* **237**; 99-101.

Cavier, R., Savel, J. and Monteoliva M. 1958. Nature et repatition, selon le sexe, des substances lipidiques chez, *Ascaris lumbricoides*, Linne' 1758. *Bull. Soc. Chim. Biol.* **40**:177-187.

Chappel L.H., 1979. Physiology of parasites. John Wiley and Sons, New York, pp178.

Chen H.Y., 1986. Absorption and retention of dietary fatty acids by marine shrimp Penaeus monodon. Paper presented at the ROC-RSA symposium. Aquaculture Turkey, Taiwan. September 5-7.

Chen H_Y., 1993. Recent advances in nutrition of *Penaeus monodon*. World Aquacult. Soc. **24(2)**: 231-245.

Chen H.Y. and Tsai R.H., 1986. The dietary effectiveness of *Artemia nauplii* and microencapsulated foods for postlarval *Penaeus monodon*. Pages 73-39 in J.L. Chuang and S.Y. Shiau (eds.) Research and development of aquatic and animal feeds in Taiwan, Vol.1. F.S.T. Monograph series No. 5. Fisheries Society of Taiwan, Taipei.

Chen H.Y., Leu T.Y. and Roelants I., 1992. Determination of quantitative arginine requirement of juvenile marine shrimp *Penaeus monodon* using microencapsulated arginine. *Marine Biology* **114**:224-233.

Cheng R. and Samoiloff M.R., 1971. Effects of cyclohexamide and hydroxyurea on mating behaviour and its development in the free-living nematode *Panagrellus silusiae* (de Man 1913) Goodey, 1945. *Can. J. Zool.* **50**:333-336.

Cheng A.C., and Briggs G.M., 1983. A study of mineral requirements in *Caenorhabditis elegans*. *Nematol.***29**.

Chin, C.H. and Bueding, E. 1954. Occurrence of oxidative phosphorylation in the muscle of *Ascaris lumbricoides*. *Biochim. Biophys. Acta.* **13**:331-337.

Chitwood B.G.,1933. A revised classification of the Nematoda. *J. of Parasitol.* **20**:131.

Chitwood B.G. and WehrE.E., 1934. The value of cephalic structure as characters in nematode classification with special references to the super family Spiruroidea. *Z.parasit.* **7 (32)**:273-338.
Chitwood B. G.,1937. A revised classification of the nematoda. Papers on Helminthology in commemoration of the 30 year Jubdeum of KI. Skojabin and the all union institute of Helminthology, Moscow. pp.69-80.

Chitwood B.G. and Chitwood H. B., 1950. An introduction to Nematology. Baltimore U.S.A. *Monumental Printing co.* pp213.

Clark W.C., 1961. A revised classification of the Order Enoplida (Nematoda). New Zealand *J. Sci.* **4**:123-150.

Colvin, P.M. 1976. The effect of selected seed oils on the fatty acid composition and growth of *Penaeus indicus*. *Aquaculture,* **8**: 81-89.

Connick Jr.W.J., Daigle D.J. and Quimby Jr. P.C., 1991. An improved invert emulsion with high water retention for Mycoherbicide delivery. *Weed Technology* **5**:442-444.

Cook H.L. and Murphy M.A., 1969. The culture of larval Penaeid Shrimp. *Transactions of the American Fisheries Society.* **98**:751-754.

Cooper A.F. and Van Grundy S.D., 1970. Metabolism of glycogen and neutral lipids by *Aphlenchus avenae* and *Caenorhabditis sp.* in aerobic microaerobic and anaerobic environments. *J. Nematol.* **2:**305-315.

Cowey C.B. and Forster J.R.M., 1971. The essentail amino acid requirement of the prawn *Palaemon serratus*. The growth of prawns on diets containing proteins of different amino acid compositions. *Mar.Biol.* **10**:77-81.

Cowey C.B., Owen J.M., Adron J. W. and Middleton C., (1976). Studies on the nutrition of marine flatfish. The effect of different dietary fatty acids on the growth and fatty acid composition of turbot (*Scopthalamus maximus*). *Br.J.Nutr.* **36**, 479-486.

Croften H.D., 1971. Form function and behaviour in plant parasitic nematodes. Zuckerman B.M., mai W.F. and Rohde R.A. (eds.). Vol. 1 83-113pp. Academic Press, New York.

Cryan W.S., 1963. A method of axenising large numbers of nematodes. *J. Parasitol.* **49**:351-352.

Curran J., Baillie, D. L., and Webster, J. M., 1985. Use of restriction fragment length differences in genomic DNA to identify nematode species. *Parasitology*, **90**:137, 1985.

Dabrowski K., 1984. Influence of initial weight during the change from live to compound feed on the survival and growth of four Cyprinids. *Aquaculture*, **25**:25-33.

Dall W. 1967. The functional anatomy of the digestive tract of a shrimp *Metapenaeus bennettae* Racek and Dall (crustacea: Decapoda: Penaeidae). *Austral. J. Zool.*. **15**:699-714.

Davenport H. E. 1949. The Haemoglobin of *Ascaris lumbricoides. Proc.Roy. Soc. B.* **136**:255-270.

Deru, J. 1990. Studies on the development and nutrition of the caridean prawn, *Macrobrachium rosenbergii* (De man) (Crustacea: Decapoda). Ph.D. thesis. University of Wales, Bangor, UK. 306 pp.

Deshimaru O. and Kuroki K., 1975. Studies on a purified diet for prawn-V. Evaluation of caesin hydrolyzates as a nitrogen source. *Bulletin of the Japanese Society of Scientific Fisheries.* **41**:301-304.

Deshimaru O. and Yone Y., 1978. Effect of dietary carbohydrate source on the growth and feed efficiency of prawn. *Bull. Jap. Soc. Sci. Fish.* **44**:1161-1163.

Deufel J., 1965. Pigmentierungsversuche mit canthaxanthin bei rigenbogenforellen. *Arch.Fischereiwiss.* **16**(2):125

Deufel J., Schweiz. Z., 1975. *Hydrologie* **37**:244.

Dougherty E.C. and Calhoun H.G., 1948. Experiences in culturing *Rhabditis pellio* (Schneider, 1866) Butschli, 1873 (Nematoda:Rhabditidae) and related soil nematodes. *Proc.Helminth.Soc.Wash.* **15**:55-68.

Doughherty G.C. 1953. Axenising and monxenising soil nematodes. *J. Parasitol.***39**:32-33 (supplement).

Dougherty G., 1959. Introduction to axenic cultures of invertebrates Metazoa; a goal. *Ann. N.Y. Acad. Sci.* **77**:27-54.

Dougherty E.C., 1960. Cultivation of aschelminthes especially rhabditid nematodes. In: Nematology. (Eds) J.N. Sasser and W.R. jenkins. Chapel Hill, University North Carolina Press. 297-318.

Duggal C.L., 1978. Sex attraction in the free - living nematode *Panagrellus redivivus. Nematol.* **24**:213-221.

Duggal C.L., 1978. Copulatory behaviour of male *Panagrellus redivivus. Nematol.* **24**:257-268.

Duggal C.L., 1978. Initiation of copulation and its effects on oocyte production and life span of adult female *Panagrellus redivivus. Nematol.* **24**:269-276.

Dutky, S.R., Robbins, W.E., and Thompson, V.V., 1967. The demonstration of sterols as requirements for the growth, development and reproduction of the DD-136 nematode, *Nematologica*, **13**:140.

Ehrlich, K.F., M.-C. Cantin and M.R. Rust. 1989. Growth and survival of larval and post larval small mouth bass fed a commercially prepared dry feed and/or *Artemia* nauplii. *Journal of the World Aquaculture Society* **20**:1-6.

Ellenby C., 1964. Haemoglobin in the 'Chromotrope' of an insect parasitic nematode. *Nature, London* **202**:615-616 (34).

Ellenby C. and Smith L., 1966. Observations on *Panagrellus redivivus*. Goodey, 1945. *J.Helminthology.* **40:**323-330.

Emmerson, W.D. 1980. Ingestion, growth and development of *Penaeus indicus* as a function of *Thalassiosira weissflogii* cell concentration. *Mar. Biol.* **58**: 65-73.

Emmerson, W.D. 1984. Predation and energetics of Penaeus indicus (decapoda: Penaeidae) larvae feeding on *Brachionus plicatilis* and *Artemia* nauplii. *Aquaculture*, **38**: 201-209.

Evans A.A.F. and Fisher J.M., 1969. Some factors affecting the number and size of nematodes in populations of *Aphelenchus avenae*. *Nematol.* **16**:295-304.

Fairbairn D., 1954. The metabolism of *Heterakis gallinae* II Carbon dioxide fixation. *Exp.Parasitol.* **3**:52-63.

Feder W.A., 1958. Aseptic culture of the burrowing nematode *Radopholus sirrulus* (Cobb). *Phytopathology*:**48**:392-393.

Fegan D.F., 1992. Recent developments and issues in the penaeid shrimp hatchery industry. In; Wyban J. (Ed.), Proceedings of the special sessions on shrimp farming, Orlando, Florida, USA, May 22-25, 1992. *World Aquacult. Soc.*. pp55-70.

Filhol, J. 1937. Phenomenes cytologiques de la secretion d'ascardine. Prteine intracellulaire des spermatozoides chez *l'ascaris megalocephala*. *Arch. Anat. Micr.* **33**:301-312.

Filipjev I., 1934. The classification of the free-living nematodes and their relation to the parasitic nematodes. *Smithsonian miscellaneous collection.* **89 (6):**1-63.

Finney J.R., 1983. Package and transportation of nematodes. *US Patent No.* A01K67/00.

Fletcher C.L. and Krusberg L.R., 1973. Investigation of some lipids from *Turbatrix aceti. Comp.Biochem.Physiol.* Vol. **45B**:159-165.

Flury, F. 1912. Zur chemie und toxikologie der ascaridien. *Arch. Exp. Path. Pharmak.* **67**: 275-392.

Folch, J., Lees, M. and Sloane-Stanley, G.H. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J.Biol. Chem.* **226**: 497-509.

Fontaine C.T., Revera D.B., Morales H.M. and Mock C.R., 1982. Observations on the mass propagation of the common soil nematode *Panagrellus redivivus* for use as foodstuff in penaeid shrimp hatcheries. NOAA Technical Report; National Marine Fisheries Service, Galveston laboratory, Galveston, Texas, U.S.A.

Foster J. and Gabbott P. 1971. The assimilation of nutrients from compounded diets by the *prawns Palaemon serratus* and *Pandalus platyceros*. J. Mar. Biol. Ass. **51**:943-961.

Fravel D.R., Marvis J.J., Lumsden R.D. and Connick Jr.W.J., 1985. Encapsulation of potential biocontrol agents in an alginate clay matrix. *Phytopathology* **75**:774-777.

Friedman M. J., 1989. Mass production in liquid culture of insect killing nematodes. *Biosys (US) Patent No.* PCT/US88/04124.

Fujii M., Nakayama H. and Yone Y. (1976). Effect of w3 fatty acids on growth, feed efficiency and fatty acid compostion of red sea bream (*Chrysophys major*). *Rept. Fish. Res. Lab., Kyushu Univ.* No.3, 65-86.

Fujii M. and Yone Y., (1976). Studies on the nutrition of red sea bream-XIII. Effect of dietary linolenic and w3 polyunsaturated fatty acids on growth and feed efficiency. *Bull. Japan. Soc. Scient.Fish.* **42**, 583-588.

Funk V.A. & Hobson L.A., 1991. Temporal variations in the carotenoid composition and content of *Euphausia pacifica* Hansen in Saanich Inlet, British Columbia. *J. Exp. Mar. Biol. Ecol.* **148**:93-104.

Fujita S., Watanabe T. and Kitajima G., 1980. Nutritional quality of *Artemia* from different locations as a living feed from the view point of essential fatty acids for marine fish. p.227-290. In: The Brine Shrimp *Artemia*, Vol.3. Ecology, Culturing Use in Aquaculture. Persoone G.,Sorgeloos P., Roels O. and Jasper E. (eds.). Universal Press, Wetteren, Belgium. 456p.

Galgani, M-L. and Aquacop. 1988. Essais de substitution des algues vivantes par des microparticules inertes pour l'alimentation des larves zoe des crevettes peneides. *Aquaculture*, **69**: 115-127.

Gbewonyo, K., Rohrer S.P., Lister L., Burgess, B., Cully, D. and Buckland,B., 1994. Large scale cultivation of the free living nematode *Caenorhabditis elegans*. *Bio/Technology* Vol.**12**:51-54

Gerlach S.A. and Schrage M., 1971. Life cycles in marine meiobenthos. Experiments at various temperatures with *Monhystera disjuncta* and *Theristus pertenuis* (Nematoda). *Mar.Biol.* **9:**274-280.

Georgis R., 1990. Formulation and application technology . In Entomopathogenic nematodes in Biological Control. Eds. R. Gaugler and H.K. Kaya. CRC Press, Boca Raton, Florida, USA. 173-191.

Glaser, R. W. 1940. The bacteria-free culture of a nematode parasite. *Proc. Soc. Exper. Biol and Med.* **43**:512-514.

Godliman J., 1983. Unpublished data.

Godliman J., 1991. March 1991 monthly report. Agricultural Genetics Company, MicroBio Ltd. U.K.

Godliman J. and Shepherd M., 1992. August 1992 monthly report. Agricultural Genetics Company, MicroBio Ltd., U.K.

Golden J.W. and Riddle D.L., 1983. The *Caenorhabditis elegans* dauer larva; Developmental effects of pheromone, food and temperature. *Dev.Biol.*, **102**:368-378.

Golden J.W. and Donald L.R., 1984. A *Caenorhabditis elegans* dauer inducing pheromone and an antagonistic component of the food supply. *J.Chem.Ecol.* **No.8, 10**:1265-1280.

Gomez R., Macarulla J.M., Garate A.M., Barbon P.G., and Milicua J.C.G., 1986. Studies on the reconstitution of *P. clarkii* blue carotenoproteins for its isolated subunit forms. *Experentia* **42**:1063-1064.

Grewal P.S., 1991. Influence of bacteria and temperature on the reproduction of *Caenorhabditis elegans* (Nematoda:Rhabditidae) infesting mushroom (*Agaricus bisporus*). *Nematologica* **37**:72-82.

Grewal P.S., Gaugler R., Kaya H.K.and Wusaty M., 1992. Infectivity of entomopathogenic nematode Steinernema scapterisci (Nematoda:Steinernematidae). J. Invert. Path..

Guillame J., 1990. The nutritional requirements of the Japanese prawn *Penaeus japonicus*. In: Barret, J. (Ed.), Advances in Tropical Aquaculture, Act. Coll. (, IFREMER, Plouzane, France. pp. 381-393

Hansen E.L. and Cryan S.W., 1966. Continuous axenic culture of free-living nematodes. *Nematol.* **12**:143-146.

Hansen H.L. and Buecher E.J., 1970. Symposium: Current approaches to the problems of systematic nematology; Biochemical approach to systematic studies with axenic nematodes. *J.Nematol.* **No.1,2**:1-6.

Hanson J.A. and Goodwin H.L., 1977. Shrimp and Prawn farming in the western hemisphere. Dowden, Hutchinson and Ross Inc. Strousburg, Pennsylvania, U.S.A.

Hard J.J. and Wethmeimer A.C. and Johnson W.F., 1989. Geographic variation in the occurence of red and white fleshed Chinook Salmon. (*Oncorhynchus tshawytscha*) in western North America. *Can.J.Fish.Aquat.Sci.* **46**:1107-1113.

Harris R.P. and Paffenhofer, G.A. 1976. Feeding, growth and reproduction of the marine planktonic copepod *Temora longicornis*(muller). *J. Mar. Biol. Ass.*. UK. **56**:675-690.

Helfrich P., 1973. The feasibility of brine shrimp production in Christmas Island. Sea Grant technical Report UNIHI-Seagrant-TR-73-02. pp173.

Hirayama O. and Kishida T., 1991. Temperature induced changes in lipid molecular species of a thermophilic cyanobacterium *Mastigocladus labinosus*. *Agricult. Biol. Chem.* **55:**781-785.

Hofsten A.V. et al., 1983. Digestion of free-living nematodes fed to fishes. *J.Fish Biol.* **23**:419-428.

Holt, G.J. 1991. Larval nutrition and feeding in closed, recirculation culture systems. page 172 in P. lavens, P. Soorgeloos, E. Jaspers and F. Ol-levier, editors. Larvi '91 Symposium in fish and crustacean larviculture. European Aquaculture Society, Gent, Belgium.

Howell, B.K. and Matthews, D., 1991. The carotenoids of wild and blue desease affected farmed Tiger shrimp (*Penaeus monodon*, Fabricus). *Comp. Biochem. Physiol.* Vol. **98B,** No. 2/3, pp. 375-379.

Hudinaga M., 1942. Reproduction development and caring of *Penaeus japonicus* Bate. *Jap. J. Zoo.* **10**:305-393.

Hudinaga M., 1967. Kurama shrimp (*Penaeus japonicus*) cultivation in Japan. FAO Fisheries Report **57**:811-832.

Hyman L.H., 1951. The Invertebrates: Acanthocephala, Aschelminthes and Entoprocta. The Pseudocoelomate Bilatria, Vol III, New York. McGaw-Hill Book Comp. Inc.

ItoT.,1960. On the culture of the mixohaline rotifer *Brachionus plicatilis* O.F. Muller, in sea water. *Rep.Fac.Fish., Prefect.Univ.Mie.* **3**:708-740.

Ivleva I.V., 1969. Mass cultivation of invertebrates, Biology and Methods. Academy of Sciences in the U.S.S.R. All Union Hydrobiological Society (transl from russian by A. Mercado, 1973, Ketør Press, Jerusalem, pp.148.

Janse J.D. and Smits P.H., 1990. Whole cell fatty acid patterns of *Xenorhabdus* species. Letters in Applied Microbiology, **10**:131-134.

Jensen P., 1978. Revision of Microlaimidae, erection of Molgolaimidae family and remarks on the systematic position of *Paramicrolaimus*. (Nematoda, Desmorida). *Zoologica scripta.*

Johns D.M., M.E. Peters and Beck A.D., 1980. International Study on Artemia VI. Nutritional value of geographical and tempporal strains of *Artemia*: Effects on survival and growth of 2 species of Brachyuran larvae. pages 290-304 in G. Persoone, P. Sorgeloos, O. Roels, and E. Jaspers (eds.), The Brine Shrimp Artemia, Vol.3. Ecology, Culture, Use in aquaculture. Universa Press, Wetteren, Belgium.

Jones D.A., Jawed T. and Tily P., 1972. The acceptance of artificial particles by Planktonic Crustacea. *Chemosphere*, **8**:133-136.

Jones D.A., Kanazawa A. and Abdel Rahman S., 1979a. Studies on the presentation of Artificial diets for rearing the larvae of *Penaeus japonicus* Bate. *Aquaculture*, **17**:33-43.

Jones D.A., Kanazawa A. and Ono, K., 1979b. Studies on the nutritional requirements of the larval stages of *Penaeus japonicus* using microencapsulated diets. *Mar.Biol* **54**, 261-267.

Jones D.A., Amjad S. and Chitravadivelu K., 1989. Comparison of artificial feeds used in penaeid shrimp hatcheries. prc. 3rd Egyptian-British Conference on Animals, Fish and Poultry. Alexandria, Egypt 7-10. October 1989. pp. 15-20

Jones, D.A., Kurmaly, K. and Arshad, A. 1987. Penaeid shrimp hatchery trials using microencapsulated diets. *Aquaculture*, **64**: 133-146.

Jones, D.A. and Kurmaly, K. 1987. Feeding and assimilation of artificial feeds by crustacean larvae. *J. World Aquacult. Soc.* **18**(1):56-57.

Jones, D.A., L.Le Vay and Mohd. Salleh Kamarudin. 1992. Feeding and nutritional requirements of penaeid shrimp larvae. *Memorias I Congresso Ecuatoriano de Acuicultura.* 45-52.

Jones D.A., Salleh Kamarudin Mohd. and Le Vay L., 1993. The potential for replacement of live feeds in larval culture. World Aquacult. Soc. June Vol.**24**, No.2 pp.199-210.

Jones D.A. 1988. Tropical prawn culture. University of Wales, Review Science and Technology, **4**: 51-58.

Jones, D.A., Kamarudin, M.S. and Le Vay, L. 1993. The potential for replacement of live feeds in larval culture. *J. World Aquacult.* Soc. **24**(2): 199-210.

Kahan D. and Appel Z., 1975. The value of *Panagrellus sp.* (Nematoda) as food for fish. *10th European Symposium on Marine Biology, Ostend, Belgium, Sept.* **17-23**, **1**:243-253.

Kahan D., 1979. Food organisms for rearing fry of Mullet In : E.A. Huisman and Hogendoorn H., (eds.). EIFAC Workshop in mass rearing of fry and fingerlings of fresh water fishes. EIFAG T.35 Suppl. I. Ministry of Agriculture and Fisheries. The Hague, Netherlands. pp. 189-193.

Kahan D., Katznelson R. and Bar-EI T., 1983. Digestion of free-living nematodes fed to fish. *J.Fish Biol.* **23**:491-428.

Kahan D., 1984. Overview:Food for larval stages in Aquaculture-Live or artificial food? *Isr.J.Zool.* **33**:152-153.

Kahan D., bar-El, T., brandstein, Y., Rigbi, M. and Oland, B. 1980. 1980. Free-living nematodes as a dietary supplement in the rearing of fish fry and hatcheries. *General Fisheries Council Mediterranean Studies review.* **57**:67-78.

Kanazawa A., 1984. Nutrition of penaeid prawns and shrimps. In: Taki, Y., Primavera, J.H. and Llobrera, J.A. (eds), Proc. 1st. Int. Conf. on Culture of Penaeid Prawns/ Shrimps, Iloilo City, Philippines, Aquaculture Department SEAFDEC. pp. 123-130.

Kanazawa A., Teshima S. and Tokiwa S., 1977. Nutritional requirements of prawn. VII. Effect of dietary lipids on growth. *Bull.Jpn.Soc.Fish.*, **43**(7):849-856

Kanazawa A., Teshima S.I. and Ono K., 1979. Relationship between essentail fatty acid requirements of aquatic animals and the capacity for bio-conversion of linolenic acid to highly unsaturated fatty acids. *Comp.Biochem.Physiol.*B, 295-298.

Kanazawa A., Teshima S., Sakamoto M. and Awal Md. A.,(1980). Requirement of *Tilapia zillii* for essential fatty acids. *Bull. Japan. Soc.scient. Fish.* **33**, 47-55.

Kanazawa, A. 1985. Microparticulate diets. In: Yone, Y. (Ed.) Fish Nutrition and Diets. Koseisha-Koseikaku, Tokyo. pp.99-110.

Kanazawa A., 1990. Microparticulate feeds for penaeid larvae. *Aquacop Ifremer Actes de Colloques* No. **9**:395-404.

Kaya H.K. and Nelsen C.E., 1985. Encapsulation of Steinernematid and Heterorhabditid nematodes with calcium alginate, a new approach for insect control and other applications. *Environ.Entomol,* **14**:572.

Kaya H.K., Choo H.Y. and Burlando T.M. Influence of plant roots on the infectivity of *Heterorhabditis heliothidis* to insect hosts. *J.Nematol.* **19**:537.

Kepner R.L. and Yu S.J., 1987. Development of a toxic bait for control of mole crickets Orthoptera, Gryllotalpidae. *J.Econ.Entomol.* **80**:659.

Khomutov G., Fry I.V., Huflejt M.E. and Packer L., 1990. Membrane lipid composition, fluidity and surface charge changes in response to growth of the fresh water cynobacterium Synechoccus 6311 under high salinity. *Archives of Biochemistry and Biophysics.* **277**:263-267.

Kinne O., 1960. Growth, food intake and food conversion in euryplastic fish exposed to different temperatures and salinities. *Physiol.Zool.* **33:**288-317.

Klass M., 1976. Non-aging development variant of *Caenorhabditis elegans. Nature*, **260**:523-525.

Klein M.G., 1990. Efficiency against soil inhabiting insect pests. In entomopathogenic nematodes in biological control. R. Gaugler and H.K. Kaya (eds.) CRC, Boca, Raton, Florida, pp195-214.

Kochs W., 1895. Experiments with artificial propagation of minute crustacea. *U.S. Fish Commission Bulletin (1894),* 306-308.

Kour V.R.D. and Subramoniam, T., 1992. Carotenoid metabolism during embryonic development of a marine crab, *Emerita asiatica* (Milne - Edwards). *Inverterate Reproduction and Development,* **21(2)**: 99-106.

Krinsky N.I., 1989. Carotenoids and cancer in animal models. Symposium on the biological actions of carotenoids 72nd annual meeting of The Federation of American Societies for Experimental Biology, Las Vegas May 2 1988 pp. 123-126.

Krusberg L.R., 1967. Analysis of total lipids and fatty acids of plant parasitic nematodes and host tissues. *Comp.Biochem.Physiol.* **21**:83-90.

Krusberg L.R., 1971. Chemical composition of nematodes In: Plant Parasitic Nematodes Ed. B.M. Zuckerman, W.F. Mai and R.A. Rhode Academic Press, London and New York Vol **11**:213-234.

Krusberg L.R., 1972. Fatty acid composition and it's culture medium. *Comp. Biochem. Physiol.* **41B**:89-98.

Krusberg L.R., Hussey P.S. and Fletcher C.L., 1973. Lipid and fatty acid composition of females and eggs of *Meloidogyne incognita* and *M. avenae. Comp. Biochem. Physiol.* **45B**:335-34.

Kuban F.D., Addison L.L. and Wilkenfeld J.S., 1985. Survival, metamorphosis and growth of larvae from four species fed six food combinations. *Aquaculture*, **47**:151-162.

Kumarly K., 1989. Studies on the acceptability and digestibility of artificial diets by Crustacea. PhD. thesis. University of Wales, Bangor, UK. 203pp.

Kurmaly K., Yule A.B. and Jones D.A., 1989. An energy budget for the larvae of *Penaeus mondon* (Fabricus). *Aquaculture*, **92**:207-218.

Kumlu M. 1995. Physiology of decapod crustacean larvae with special reference to diet. Ph.D thesis. University of Wales, Bangor, UK.

Kumlu M. and Jones D.A., 1995. The effect of live and arificial diets on growth, survival, and trypsin activity in larvae of *Penaeus indicus*. World Aquacult. Soc.. **26, No.4**, December 1995:406-415.

Kumlu M. and Jones D.A., 1997. Digestive protease activity in planktonic crustaceans feeding at different trophic levels. *J.Mar Biol.Ass. UK.* **77**:159-165.

Kuo H.S., Lee T.C., Chichester C.O. and Simpson K.L., 1976. The carotenoid in the deep sea red crab *Geryon quiquedens. Comp. Biochem Physiol.* **54B**: 387-390.

Kuronuma K. and Fukusho K., 1984. Rearing of marine Fish Larvae in Japan. IDRC, Ottawa, Ont 109pp.

Kurmaly K., Jones D.A., Yule A.B. and East J., 1989. Comparative analysis of the growth and survival of Penaeus monodon (Fabricus) larvae, from protozoea 1 to postlarva 1, on live feeds, artificial diets and on combinations of both. *Aquaculture*, **81** (1989) 27:45.

Lackey B.A., Muldoon A.E. and Jaffe B.A. (1993). Alginate pellet formulation of *Hirsutella rhossiliensis* for biological control of Plant Parasitic Nematodes. *Biological Control* **3**:155-160.

Langdon, C.J., Levine, D.M. and Jones, D.A. 1985. Review: microparticulate feeds for marine suspension feeders. *J. Microencapsulation*, **21**(2): 1-11.

Larsen E., 1941. 'Er Mikroorm endansk Opdagelse?' *Akvarie.vennen.Helsingør.* 2-4

Lee D.L., 1966. An electron microscope study of the body wall of the third stage larva of *Nippostrongylus braziliensis. Parasitol.* **56**:127-135.

Lee D.L. and Atkinson H.J., 1977. Physiology of Nematodes. Columbia University Press, New York, 283pp.

Lee, R.G., Neamtu, G.Gh., Lee, T.C., and Simpson, K.L., 1978. Pigmentation of rainbow trout with extracts of floral parts from *Tagetes erecta* and *Curcubita maxima marcia*, *Rev. Roum. Biochem.*, **15 (4)**:287.

Leger, Ph., Bieber, G.F. and Sorgeloos, P. 1985. International study on Artemia XXXIII. Promising results in larval rearing of *Penaeus stylirostris* using prepared diet as algal substitute and for *Artemia* enrichment. *J. World Maricult. Soc.* **16**: 354-367.

Leger, Ph., Bengtson, D.A., Sorgeloos, P., Simpson, K.L. and Beck, A.D. 1986. The use and nutritional value of *Artemia* as a food source. *Oceanogr. Mar. Biol. Ann Rev.* **24**:521-623.

Leger, Ph. and Sorgeloos, P. 1992. Optimised feeding regimes in shrimp hatcheries. In: Fast, A.W. and Lester, L.J. (Eds.), Marine Shrimp Culture: Principles and Practices, Elsevier. pp. 225-244.

Le Vay L., 1994. Nutritional studies on fish and crustacean larvae. PhD. thesis. University of Wales, Bangor, UK. 132 pp.

Le Vay, L., Rodriguez, A., Kamarudin, M.S. and Jones, D.A. 1993. Influence of live and artificial diets on tissue composition and trypsin activity in *Penaeus japonicus* larvae. *Aquaculture*, **118**:287-297.

Lewis J.A. and Papavizas G.C., 1987. Application of *Trichoderma* and *Gliocladium* in alginate pellets for control of *Rhizoctonia* damping off. *Plant.Pathol.* **36**:438-446.

Liao, I. C., Su, M. H. and Lin, J. H. 1983. Larval foods for penaeid prawns. In: Mcvey, J.P. (Ed.), CRC Handbook of Mariculture, Vol. 1. CRC press, Boca Raton, FL. pp. 43-70.

Liao, I.C. 1984. Status and problems of grass prawn culture in Taiwan. In: Liao, I. C. and Hirano, R. (Eds.), TML Conf, Proc., No. 1, Tungkang Marine Laboratory, Tungkang, Taiwan. pp.81-98.

Liao T.C., 1984. A brief review of the larval rearing techniques of penaeid prawns. Proceedings of the First International Conference on the culture of Penaeid Prawns/Shrimps. **1**:65-78.

Liao, I.C., Kumeno, F., Iida, Z. and Kobayashi, T. 1988. Preliminary report on the application of artificial plankton B.P. in *Penaeus monodon* larval production. *J. World. Aquacult. Soc.* **19**:47A.

Liao I.C. and Lui F.G., 1990. A brief review of nutritional studies for penaeus monodon. In: Barret, J. (Ed.),Advances in Tropical Aquaculture, act. Coll, IFREMER, Plouzane, France. pp.355-380.

Liu, L. X and Weller, P. F. 1989. *Brugia malayi* micofilarial polyunsaturated fatty acid composition and synthesis. *Exp. Parasitol.* **47**: 319-328.

Lorentz L.Ya., 1960. Nematodes as food for fry of aquarium fishes. proceedings of the Vth Soviet Union Congress for Research on nematodes, Samarkland, State University. 59-60 (In Russian).

Lorenzen S., 1981. Eutwurf eines phtogenetischen system der freilebenden Nematoden. Veroffentlichen. *Institute fur Meeresforsehung Bremerhaven Suppl* **7** pp 449.

Lovett, D.L. and Felder, D.L. 1988. Evaluation of the rotifer, *Brachionus plicatilis* as a substitute for *Artemia* in feeding larvae of *Macrobrachium rosenbergii* larvae. Aquaculture, 71:331-338.

Lovett, D.L. and Felder, D.L. 1989. Ontogeny of gut morphology in white shrimp *Penaeus setiferus* (Decapoda; Penaeidae). *J. Morphology*, **201**: 253-272.

Lovett, D.L. and Felder, D.L. 1990a. Ontogenetic change in digestive enzyme activity of larval and post larval white shrimp, *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). *Biol. Bull.* **178**: 144-159.

Lovett, D.L. and Felder, D.L. 1990b. Ontogenetic changes in enzyme distribution and midgut function in development stages of *Penaeus setiferus* (Crustacea, Decapoda, Penaedae). *Biol. Bull.* **178**: 160-174.

Lower W.M.R. and Buecher E.J., 1970. Axenic culturing of nematodes: An easily prepared medium containing yeast extract. *Nematol.*, **16**:563-566.

Marte, C.L. and M.N. Duray. 1991. Microbound larval feed as supplement to live food for milkfish (*Chanos chanos* Forsskal) larvae. Pages 175-177 in P. Lavens, P. Sorgeloos, E. jaspers and F. Ol-levier, editors. Larvi'91 Symposium on fish and crustacean larviculture. European Aquaculture Society, Gent Belgium.

Matsuoka T., 1975. Culture of the giant prawn. *Yoshuku* **12**:48-52.

Meyers S.P. (1977). *Feedstuffs* **43** (**19**):26-28.

Mikulin A.Y., Soin S.G., 1975. The functional significance of carotenoids in the embryonic development of teleosts. *J. Ichthy.* **15**:749-759.

Mock C.R. and Neal R.A., 1974. Penaeid shrimp hatching systems. Proceedings of the FAO CARPAS Symposium on Aquaculture, Montevideo, Uruguay, 26 November - 2 December 9pp.

Moore P.G., 1971. The nematode fauna associated with holdfasts of kelp (*Laminaria hyperborea*) in North-East Britain. *J. Mar. Biol. Ass.* UK. **51**:589-604.

Morrison, W.R. and Smith, L.M. 1964. Preparation of fatty acid methyl esters and dimethylacetate from lipids with boron flouride methanol. *J. Lipid. Res.* **5:** 600-608.

Munilla-Moran, R.. and J.R. Stark.. 1989. Protein digestion in early turbot larvae, *Scopthlalmus maximus* L. *Aquaculture* **81**:315-327.

Munilla-Moran, R., J.R. Stark and A. Barbour., 1990. The role of exogenous enzymes in digestion in cultured turbot larvae (*Scopthalmus maximus* L.). Aquaculture 88:337-350.

Myers R.F., 1968. Nutrient media for plant parasitic nematodes: 1. Axenic cultivation of *Aphelenchoides sp. Exp. Parasitol.* **23**:96 - 103.

Nelsen et al.,1986. United States patent : Hydrogel encapsulated. nematodes No. 4, 615883 oct 7 1986.

New, M.B. 1976. A review of dietary studies with shrimp and prawns, *Aquaculture*, **9**:101-144.

New, M.B. 1980. A Bibliography of shrimp and prawn nutrition. *Aquaculture*, **21**:101-128.

Nicholas W.L., Dougherty E.C. and Hansen E.L., 1959. Axenic cultivation of *Caenorhabditis briggsae* (Nematoda:Rhabditidae) with chemically undefined supplements;comparative studies with related nematodes. *Ann.NY.Acad.Sci.* **77**:218-236.

Nicholas W.L., 1962. A study of a species of Acrobeloides (Cephalobidae) in laboratory culture. *Nematol.* **8**:99-109.

Nicholas W.L., 1975. The biology of free-living nematodes. Oxford: Clarendon Press.

Nicholas W.L., 1984. The biology of free-living nematodes, 2nd ed. Clarendon Press, Oxford, U.K.

Nigon V., 1949. Modalities de la reproduction et determine du sexe chez quelques nematodes libres. *Annls Sci Nat.* **11**:1-42.

Nikolsky G.V., 1963. The ecology of Fishes (translated by L. Birkett) London:Academic Press.

Okimasu, E., Matsumoto M., Yoshida Y., Amemura, A., 1992. The effects of the pigments of *Rhodobacter capsulatus*. on free radicals and application of the bacterium as a feed to larval fish larvae. *Bull. Jap. Soc. Sci. Fish.*, **58(8)**: 383-385.

Omara-Alwala T.R., Chen H.M., Ito Y., Simpson K.L. and Meyers S.P., 1985. Carotenoid pigments and fatty acid analyses of crawfish oil extracts. *J.Agric.Food.Chem.* **33**:260-263.

Opuszynski K., 1977. Rearing of larvae and fry of silver carp, *Hypophthalmichthys molitrix*. Polish Hydrobiological Society 'Conference on Aquaculture in cultivation of fish fry and it's live food' Szymbar K., Poland, September 1977.

Ottogali L., 1991. Total substitution of microparticules for algae for Penaeus stylirostris larval rearing in New Caledonia. *Journal of the World Aquaculture Society* 22:46A.

Owen J.M., Adron J.W., Sargent J.R. and Cowey C.B. (1975). Studies on the nutrition of marine flatfish. The effect of dietary fatty acids on the tissue fatty acids of the plaice. *Pleuronectes platessa. Mar. Biol.* **13**, 160-166.

Pace, G.W., Grote, W., Pitt, D.E. and Pitt, J. M. Biotechnology Australia Pty Ltd international patent application WO. 86/01074, 27 February 1986.

Papavizas G.C., Fravel D.R. and lewis J.A., 1987. Proliferation of *Telromyces flavus* in soil and survival in alginate pellets. *Phytopathology* **77**:131-136.

Papiz M.Z., Sawyer L., Eliopoulos E.E., North A., Findlay J., Sivaprasadavao R., Jones T.A., Newcomer M.E., Kraulis P.J. *Nature* **324**:383-385.

Pascual F.P., 1986. Effect of supplemented lecithin and lipid sources on the growth and survival of *Penaeus monodon* juveniles. Pages 615-618 in J.L. Maclean, L.B. Dizon and L.V. Mosillos (eds.). The first Asian Fisheries Forum. Asian Fisheries Society, Manilla Phillipines.

Passey, R.F. and Fairbairn, D. 1957. The conversion of fat to carbohydate during embryonation of *Ascaris* eggs. *Can J. Biochem. Physiol.* 35: 511-525.

Pasternak J., 1970. Scanning electron microscope studies on the free-living nematode *Panagrellus silusiae* (Rhabditidae). *Nematol.* **16**:375-381.

Patel T. R. and McFadden B. A., 1978. Axenic and synchronous cultures of *C. elegans*. *Nematol.* **24**:51-62.

Perry R.N. and Clarke A.J., 1981. *Parasitology.* **83**:435-449.

Petit H., Sance S., Negre-Sadargues, G., Castillo R. and Trilles J.P., 1991. Ontogeny of carotenoid metabolism in the prawn *Penaeus japonicus* Bate (1888) (Crustacea Penaeidea). A qualitative approach. *Comp. Biochem. Physiol.* Vol. **99B** No.3: 667-671.

Petriello R. P. and Myers R. F., 1971. Nutrient media for plant parasitic nematodes III. Carbohydrate investigation on *Aphelenchoides sp. Exp. Parasitol.* **29**:423-432.

Petronijevic T. and Rogers W.P., 1987. *Comp.Biochem. Physiol.* **88**A:207-212.

Phillips A.M., Neilsen R.S. and Brockway D.R., 1954. A comparison of hatchery diets and natural food. *Progressive.Fish Culturist.* **16**:153-151.

Plagman J., 1939. Ernahrungsbiologie der garnele (*Crangon vulgaris* Fabr.) helgolander wiss. Mecresunters **2**:113-162.

Platon R.R., 1978. Design, operation and economics of a small scale hatching for the larval rearing of *Penaeus monodon* Fabricus. SEAFDEC. Aquaculture Extension manual no.1 pp.30.

Platonova T. A., 1970. On the taxonomy of the family Leptosomatidae (Nematoda) from the Mediterranean and the adjacent Atlantic waters. *Zoologicheskii Zhurnal* **49**:1298-1305.

Pollock C. and Samoiloff M. R., 1976. The development of stage specific behaviour in panagrellus redivivus. *Can. J. Zool.* **54**:674-679.

Poinar G.O., Thomas G.M., Lin K.C. and Mookerjee K., 1985. Feasibility of embedding parasitic nematodes in hydrogels for insect control. IRCS *Med.Sci.* **13**:754.

Popiel R., 1987. Unpublished data.

Protasswicka A., 1985. Carotenoids in Frozen Antartic Krill and some of it's semi finished products. *Acta Alimentaria Plolnica.* **9**:235-244.

Quinito E., de la Pena D. and Pascual F. and 1983. The use of suitable feed in larval rearing of *Penaeus monodon.* pp 337-342 in Rogers G.L., Day R. and Lim A. (eds). Proceedings of the first International Conference on Warm Water Aquaculture-Crustacea. brigham Young University Hawaii Campus, Honolulu, Hawaii, U.S.A.

Radwin I.A. and Rouse D.B., 1990. Yield characteristics of the free-living nematode *P. redivivus* in different culture media. *The Progressive Fish Culturist* **52**:237-240.

Rand R.P. and Parsegian V.A., 1989. Hydration forces between phospholipid bilayers. *Biochemica et Biophysica Acta* **988**: 351-376.

Rathbone, L. and Ress, K. R. 1954. Glycolysis in *Ascaris lumbricoides. Biochem. Biophys. Acta.* **15**:126-133.

Redenbaugh K., Nichol J., Kossler M.E. and M.E. and Paasch B.,1984. Encapsulation of somatic embryos for artificial seed production. *In vitro* 20.

Rees, J.F., Cure, K., Piyatiratitivorakul, S., Sorgeloos, P. and Menasveta P. 1994. Highly unsaturated fatty acid requirements of *Penaeus monodon* post-larvae: an experimental approach based on *Artemia* enrichment. *Aquaculture*, **122**: 193-207.

Rodriguez, A., Le Vay, L., Mourente, G.and Jones, D.A. 1994. Biochemical composition and digestive enzyme activity in larvae and postlarvae of *Penaeus japonicus* during herbivorous and carnivorous feeding. *Mar.Biol.* **118**:45-51.

Roeder, M. and Roeder, R.H, 1966. Effect of iron on the growth of fishes. *J.Nutr.*, **90**: 86-90.

Rogers W.P.,1962. The nature of parasitism. The relationship of some metazoan parasites to their hosts. Academic Press new York 287p.

Rogers, W.P. and Lazarus, M. 1949. Glycolysis and related phosphorus metabolism in parasitic nematodes. *Parasitol.* **39**:302-314.

Rollefsen G., 1939. Artificial rearing of fry of seawater fish. Prelimnary Communications. *Rapp.Proc-Verb.Reun.Cons.Perm. Explor.Mar.* 109-130.

Rothstein M. and Cook E., 1966. Nematode Biochemistry-VI. Conditions for axenic culture of *Turbatrix aceti, Panagrellus redivivus, Rhabditis anomala* and *Caenorhabditis briggsae. Comp. Biochem. Physiol.*, **17**:683-692.

Rothstein M., 1968. Sterol requirement for reproduction of a free-living nematode. *Science*, **May**:778-779.

Rothstein M., 1971. Nematode Biochemistry XI. Biosynthesis of fatty acids by *Caenorhabditis briggsae* and *Panagrellus redivivus*. *Int.J.Biochem.* **1**:422-428.

Rottman R.W., 1988. Microworm culture for aquarium fish producers. IFAS fact sheet FA-9. University of Florida, Gainesville Florida, 2p.

Rottman R.W., Shireman J.V. and Lincoln E.P., 1991. Comparison of 3 live foods and two dry diets for intensive culture of grass carp and bighead carp larvae. *Aquaculture* **96**:269-280.

Rouse D.B., Webster C.D. and Radwin I.A., 1992. Enhancement of the fatty acid composition of the nematode *Panangrellus redivivus* using three different media. *Journal of the World Aquaculture Society* Vol.**23**. No.1. March 1992. 89-95.

Roy T.K., 1973. Selection of media for culturing free-living nematodes: Axenic culture of *Chiloplacus lentus* Thorne Nematoda:Rhabditidae). *Indian J. Nematol.* **3**:64-70.

Roy T.K.,1975. Effect of amino acids and carbohydrates on the population growth of *Chiloplacus lentus* in axenic culture. *Nematol.* **21**:12-18.

Rychly D., 1977. Rearing of silver and marble carps, *Hypophthalmichthys molitrix* and *Aristichthys nobilis*, with artificial feed. Ibid.

Samocha, T. and Lewinsohn C.H., 1977. A preliminary report on rearing penaeid shrimps in Israel. *Aquaculture* **10**:291-292.

Samoiloff M.R. and Pasternak J., 1968. Nematode morphogenesis:fine structure of the cuticle of each stage of the nematode, *Panagrellus silusiae*. (de Man 1913) Goodey 1945. *Can. J. Zool.*, **46**:1019-1022.

Sargent J. R. and Whittle K.J. 1981. In : Analysis of Marine Ecosystems. pp. 491-533. Academic press, London.

Sayre F.W., Hansen E.L. and Yarwood E.A., 1963. Biochemical aspects of the nutrition of *C. briggsae. Exp. Parasitol.* **13**:98-107

Seal F., 1933. Brine Shrimp (*Artemia*) as a satisfactory live food for fishes. *Trans.Am.Fish.Soc.* **63**:129-130.

Segner H., Verreth J. and Witt U., 1993. larval Nutritional Physiology: Studies with *Coregonus larvaretus* and *Scopthalamus maximus*. *Journal of the World Aquaculture society*. Vol **24**, No.2:121-134.

Selvan Sen., Gaugler R. and Grewal P.S., 1993. Water content and fatty acid composition of the infective juvenile entomopathogenic nematodes during storage. *J.Parasitol.* **79(4)**:510-516.

Schuurmans Stekhoven J. H and De Coninck L. A. P., 1933. Morpholologisches Fragen Zur Systematik der freilebenden Nematoden. *Verhandlungen der deutschen Zooloischen Gesellschaft* **35**:138-143.

Schroeder W.J., 1990. Super absorbent polymer as a survival aid to entomogenous nematodes for control of *Diaprepes abbreviatus* (*Coleoptera curculionidae*) in citrus. *J. Econ.Entomol.*

Schiedt K., Leuenberger F.J., Jicchi M and Blinz E., 1985. Absorption retention and metabolic transformation of carotenoids in rainbow trout, salmon and chicken. *Pure and Applied Chemistry* **57**:685:692.

Sheen S.S. and Chen S.J., 1992. The optimal dietary lipid requirement level of the tiger prawns *Penaeus monodon.* Paper presented at the 23rd Annual Conference of the World Aquaculture Society, Orlando, Florida. May 21-25.

Shigueno K., 1975. Shrimp culture in Japan. Association for International Technical Promotion, Tokyo pp153.

Shimizu I., Kitabate S., Kato M., 1981. *J. Insect Physiol.* **27**:593.

Shroeder W.A. and Johnson E.A., 1993. Antioxidant role of carotenoids on *Phaffia rhodozyma*. *Journal of Gene Microbiology.* **139**:907-912.

Sick L.V., and Andrews J.W., 1973. The effect of selected dietary lipids, carbohydrates and proteins on the growth, survival and body composition of *Penaeus duorarum. Proceedings of the World Mariculture Society.* **4**:263-276.

Sivapalan P. and Jenkins W.R., 1966. Phospholipid and long chain fatty acid composition of the nematode *Panagrellus redivivus. Proc.Helminth.Soc.Wash.* **33**:83-90.

Slobodkin L.B., 1968. Aspect of the future ecology. *Biol.Sci.Tokyo.* **18**:16-23.

Sorgeloos P., 1980 The use of the Brine shrimp Artemia in aquaculture. The Brine Shrimp *Artemia*. Vol.3. Ecology, Culturing, Use in Aquaculture, Persoone C., Soorgeloos P., Roels O. and Jaspers E. Universal Press, Wetteren, Belgium .

Sorgeloos P., 1979. The Brine Shrimp *Artemia salina*. p321-324 In: Advances in aquaculture. Pillay T.V.R. and W.A. Dill (eds.). Fishing News Books Ltd. Farnham England.

Sorgeloos, P., Dehasque, M., Dhert, P. and Lavens P. 1993. Review on present status of marine fish larviculture. - ICES. Symp. on Mass Rearing of Juvenile Fish.

Sorgeloos, P., Bossuyt, E., Lavens, P., Baezamezza, M. and Persoone, G. 1983. The use of brine shrimp *Artemia* in crustacean hatcheries and nurseries. In: McVey, J.P. (ed.), CRC Handbook of Mariculture, Vol. !. CRC Press, Boca Raton, FL. pp.71-96.

Southey J.F. 1985. Laboratory Methods for work with Plant and Soil Nematodes. Reference Book **402**, *Ministry of Agriculture Fisheries and Food.*

Smith B.S. et al. Cryopreservation of the entomogenous nematode parasite *Steinernema feltiae* (*N. carpocapsae*). *Biosys Patent No.* PCT/AU86/00285.

Sterba C., 1967. Aquarium care. London, Studio Vista Ltd.pp 515

Steven D.M., 1948. Studies on animal Carotenoids I. Carotenoids of the brown trout (*Salmo trutta* LINN). *Journal of Experimental Biology* **25**:369-387.

Stoll, N.R. 1953. Axenic cultivation of the parasitic nematode, *Neoplectana glaseri*, in a fluid medium containing raw liver extract. *J. Parasitol.* **39**: 422-444.

Stryer I., 1988. Biochemistry, 2nd ed. Freeman, New York, pp473.

Tanaka Y., Matsuguchi H., Katayama T., Simpson K. and Chichester C., 1976. The biosynthesis of astaxanthin. XVIII. The metabolism of the carotenoids in the prawn *Penaeus japonicus* Bate. *Nippon Suisan Gakkaishi,* **42**: 197-202.

Taylor A.L., Feldmesser J. and Fedre N.A., 1958. A new technique for preliminary screening of nematodes. *Plant Dis. Rptr.* **41**:527-530.

Teitjen J.H. and Lee J J., 1972. Life cycles of marine nematodes. *Oecologia*, **10**:167-176.

Teitjen J.H. and Lee J.J., 1973. Life history and feeding habits of the marine nematode, *Chromadora macrolaimoides* Steiner. *Oecologia,* **12**:303-314.

Teitjen J.H. et al., 1970. Gnotobiotic culture and physiological ecology of the marine nematode *Rhabditis marina* Bastian. *Limnol Oceanogr.* **15**:535-543.

Teshima S., Kanazawa A. and Kamashita M., 1986. Dietary values of several proteins and supplemental amino acids for larvae of the prawn *Penaeus japonicus. Aquaculture* **51**:225-235.

Thompson S.N., and Barlow., 1974. The fatty acid composition of parasitic Hymenoptera and its possible biological significance. Annals of the Entmomological Society of America **67**:627-632.

Thomson J.M., 1963. Synopsis of biologica data on the grey mullet *Mugil cephalus* Linnaeus 1758. CSIRO Australia. *Fish.Oceanogr.*, Fish Synopsis 1. 66pp.

Thomson J.M., 1966. The grey mullets. *Oceanogr.Mar.Biol.a.Rev.*, **4**:301-335

Thongrod. S., Tansutapanich. A., and Torrissen O.J. 1995. Effect of dietary astaxanthin supplemetation on accumulation, survial and growth in postlarvae of *Penaeus monodon* Fabricus. Larvi'95 - Fish & Shellfish Larviculture Symposium. P. lavens, E. Jaspers,and I Roelants (eds). European Aquaculture Society, Special Publication No. 24, Gent , Belgium. 1995.

Torrissen O.J., 1984. Pigmentation of Salmonids - effect of carotenoids in eggs and start feeding in survival and growth rate. *Aquaculture,* **43**:185-193.

Torrissen O.J., Hardy R.W., Shearer K.D., Scott T.M. and Stone F.E., 1990. Effects of dietary canthaxanthin level and lipid level on apparent digestibility coefficients for canthaxanthin in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, **88**. pp. 351-362.

Vallet F., Berhaut J., Leray C., Bonnetand B. and Pic P., 1970. Preliminary experiments on the artificial feeding of Mugillidae. Helgolander Wiss. Mecresunters. **20:**610-619.

Van der Wind J.J., 1979. Feeds and feeding in fry fingerlings culture. Paper presented at the 1979 FIFAC Workshop in mass rearing of fry and fingerlings of fresh water fishes, The Hague, May 1979.

Vanfleteren J. R., 1976. Large scale cultivation of the free-living nematode (*Caenorhabditis elegans). Experentia,* **32**/8:1087-1088.

Vanfleteren J. R., 1978. Axenic culture of free-living plant parasitic and insect parasitic nematodes. *Ann. Rev.Phytopathol.*, **16**:131-157.

Van Grundy S.D., Bird A.F. and wallace H.R., 1967. Aging and starvation in larvae of *Meloidogyne javanica* and *Tylenchus semipenetrans. Phytopathology* **57**:559-571.

Vitiello 1969. Hopperia nouveau genre de Nematode libre marin (Comesomatidae). *Tethys* **1**:485-491.

Walford, J. and T.J. Lam 1991. Growth and survival and seabass (*Lates calcarifer*) larvae fed microencapsulated diets alone or together with live food. Pages 184-187 in P. Lavens, P. Sorgeloos, E. Jaspers and F. Ol-levier, editors. Larvi'91 Symposium on fish and crustacean larviculture. European Aquaculture Society, Gent Belgium.

Walker H.L. and Connick W.J.Jr., 1983. Sodium alginate for production and formulation of mycoherbicides. *Weeds Sci.* **31**:333-338.

Wallace R.A., Walker S.L. and Hauschka V., 1967. Crustacean lipovitellin. Isolation and characterization of the major high density protein from eggs of Decapoda. *Biochemistry,* **6**:1582-1590.

Ward D.G., Middleditch B.S., Missler S.A. and Lawrence A.L., 1979. Fatty acid changes during larval development of *Penaeus setiferus. Proc. World Maricult. Soc.*. **10**:464-471.

Watanabe T., Oowa F., Kitajima C., Fukusho K. and Fujita S., 1978. Nutritional quality of brine shrimp *Artemia salina*, as a living feed from the viewpoint of essential fatty acids for fish. *Bull.Jpn.Soc.Sci.Fish.* **44**:1115-1121.

Watanabe T., Oowa F., Kitajima C. and Yone Y., 1979. Relationship between the dietary values of rotifers, *Brachionus plicatilis* and their content of w3 highly unsaturated fatty acids. *Bull.Jpn.Soc.Sci.Fish.* **45**:883-889.

Watanabe T., 1993. Importance of Docosahexaenoic acid in marine larval fish. *World Aquacult. Soc.*. June Vol. 24, No.2 152-161.

Watanabe, T., Kitajima, C. and Fujita, S. 1983. Nutritional values of live organisms used in Japan for mass propagation of fish: a review. *Aquaculture*, **34**: 115-143.

Watanabe T., Takeuchi T. and Ogino C., 1978a. Studies on the sparing effect of lipids on dietary protein in rainbow trout (*Salmo gairdneri*) *Proceedings of the World Symposium on Finfish nutrition and Fish feed tchnology*, Hamburg 20-23 June pp 113-125.

Wickens J.F., 1972. The food value of the brine shrimp *Artemia salina* fed to larvae of the prawn *Palaemon serratus* Pennant. *J.Exp.Mar.Biol.Ecol.* **10**:151-170.

Wijbenga J., 1991. Storage of Nematodes. E.C. Project 151. November 1992 Progress Report.

Wilkenfeld J.S., Lawrence A.L. and Kuban F.D., 1984. Survival, metamorphosis and growth of penaeid shrimp larvae reared on a variety of algal and animal foods. *J. World Maricult. Soc.*. **15**:31-49.

Wilson P.A.G., 1976. Nematode growth patterns and the moulting cycle: the population growth profile. *J.Zool.Lond.* **179**:153-151.

Womersley C., 1987. A reevaluation of stratergies employed by nematode anhydrobiotes in relation to their natural environment. In Vistas on Nematology, Veech J.A. and Dickson D.W., (Eds), Society of Nematologists, Hyattsville, pp165.

Wadsworth W.G. and Riddle D. L., 1988. Acidic intracellular pH shift during *Caenorhabditis elegans* larval development. *Proc.Natl.Acad.Sci. USA*, **85**: 8435-8438.

Watanabe T., Takashima F. and Ogino C. (1974). Effect of dietary methyl linolenate on growth of rainbow trout. Bull.Japan. Soc.sciet. Fish. **40**, 181-188.

Weiser W. 1953. Freeliving marine nematodes. I Enoploidea. Acta Universitatis Lund. (N.F.2). **49(6)**:1-155.

Weiser W. 1954. Freeliving marine nematodes II. Chromadoridea. Acta Universitatis Lund. (N.F.2) **50(16)**:1-148.

Wilson M., 1989-1990. AGC sponsored project. Biological control of slugs. Fifth progress report September 1989-February 1990. Long Ashton Research Station Research Station, Long Ashton, Avon.

Wouts, W.M. et al. (1981) *J. Nematol.* **13**:467-489.

Xu, X., Ji, W., Castell, J.D. and O'Dor, R. 1993. The nutritional value of dietary *w*-3 and *w*-6 fatty acids for the Chinese prawn (*Penaeus chinensis*). *Aquaculture*, **118**:277-285.

Yamada S., Tanaka Y., Sameshima M. and Ito Y., 1990. Pigmentation of prawn (*Penaeus japonicus*) with carotenoids. 1. Effect of dietary astaxanthin, B-carotene and canthaxanthin on pigmentation. *Aquaculture*, **87:** 323-330.

Yamaoka T., Sabuti K. and Katoh S., 1978. Preparation of thylakoid membranes active in oxygen evolution at high temperatures from thermophilic blue green alga. In Photosynthetic Oxygen Evolution. H. Metzner (ed.). Academic Press, New York, 104:116.

Yarwood E.A. and hansen E.L., 1968. Axenic culture of *Pelodera strongyloides* Scneider. *J. Parasitol.* **54, No. 1**:133-136.

Yone Y. (1978) Essential fatty acids and lipid requirements of marine fish. In Dietary Lipid in Aquaculture (Edited by Japan. Soc. Sci. Fish.), 43-59. Koseisha-Koseikaku, Tokyo.

Yufera, M., Rodriguez, A. and Lubian, L. M. 1984. Zooplankton ingestion and feeding behaviour of *P. kerathurus* larvae reared in the laboratory. *Aquaculture*, **42**: 217-224.

Yukawa T. and Pitt J., 1985. Nematodes storage and transport. US Patent No. PCT/AU85/00020.

Zimmerman A., 1921. Recherches experimentales sur l'elevage aeptique de l'anguille du vinaigre, *Anguillula oxophila* Schneider. Rev. *Suisse Zool.* **28**:357-380.