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Physiology of decapod crustacean larvae with special reference to diet.

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PHYSIOLOGY OF DECAPOD CRUSTACEAN LARVAE WITH SPECIAL REFERENCE TO DIET

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SUMMARY

This study investigates the factors affecting larval and postlarval survival and growth of some de capod crustaceans with special emphasis on diets. Investigations were concentrated on the influence o live and artificial diets on larval growth, survival, development and trypsin activity of a commercially important marine penaeid shrimp *Penaeus indicus* and a freshwater prawn *Macrobrachium rosenbergii*. In addition, feeding behaviour, gastroevacuation time, trypsin activity of other decapod species were also studied.

Live mixed microalgae *Tetraselmis chuii* and *Skeletonema costatum* at 60-70 cells μl^{-1} promoted highest larval survival, fastest growth and development in *P. indicus* in comparison to single algal species. *Rhinomonas reticulata* neither alone nor in combination with other algal species was suitable as food for the shrimp larvae. A water salinity (S) of 25 ppt was optimal for larval and postlarval culture of this penaeid species. Postlarvae (PL) of *P. indicus* reared at lower salinities between PL7 and PL60 (20-30 ppt) had a significantly ($P<0.05$) higher survival and a better growth than those at higher water salinities. Early PL resisted sudden salinity change of 10 ppt, but required an adaptation period for greater salinity changes. 10 ppt S was lethal to animals at around PL40-45.

A free-living nematode *Panagrellus redivivus* was found to be a suitable alternative for live algae and *Artemia* in the culture of *P. indicus*. The nematodes gave good survival, but lower growth than algae/*Artemia* from PZ1 to PL1. Larval growth and survival were significantly improved when the larvae were fed on either nematodes plus algal co-feeds or lipid-enriched nematodes. Pigmented- (astaxanthin) nematodes also improved survival and colour of *P. indicus* larvae in comparison to non-pigmented ones.

Conventional live diets were also completely replaced using microencapsulated diets (MED) for the culture of *P. indicus*. Like the nematodes, MED as a sole feed resulted in lower survival, slower growth and development in comparison to algae/*Artemia*. Addition of 15 cells μl^{-1} frozen algae significantly improved growth and survival during larval development. The larvae fed MED plus algal co-feeds had significantly ($P<0.05$) higher trypsin activity than those fed MED as a sole feed. Similarly, provision of 15 cells μl^{-1} algae with nematodes for only 24h or 48h resulted in significant increase in trypsin activity and improved survival and growth to levels comparable to those obtained from algae/*Artemia*. It appears that the presence of an algal diet is necessary to induce larval trypsin activity in *P. indicus* at early protozoeal stages, but algae do not influence trypsin at mysis stages. Results suggest that both nematodes and formulated diets lack gut enzyme stimulants and are less digestible than algae/*Artemia* diets. When freeze-dried algal materials were incorporated into MED, it was found that algal substances which trigger larval digestive enzymes were retained within the capsules. Whether this will improve growth and survival of penaeid larvae remains to be examined.

In contrast to penaeid larvae, a complete replacement of live *Artemia* with nematodes or artificial diets was not possible for the culture of caridean *M. rosenbergii* and *Palaemon elegans* larvae. For both species, only a partial replacement was achieved from Z4/5 to metamorphosis by using formulated diets. It was found that these larvae have very low trypsin activity levels between Z1 and Z4/5, but the levels increase sharply afterwards, coinciding with a vast increase in the hepatopancreas. This sharp increase in digestive enzyme activities and longer food retention time enable these larvae to survive on less digestible formulated diets.

A comparison of specific trypsin activity in several larval decapod crustaceans shows a pattern with high levels in herbivores, low levels in carnivores and intermediate levels in omnivores. Herbivorous penaeid larvae (*P. indicus*) and copepods (*Temora longicornis* and *Centropages typicus*) rely on high digestive enzyme activities to extract nutrients from less digestible algae, whereas carnivorous larvae the lobsters (*Homarus gammarus* and *Nephrops norvegicus*) and carideans (*M. rosenbergii* and *P. elegans*) have limited enzymatic capacity and hence require large and easily digestible prey, but resist long starvation periods. Omnivorous mysis penaeid larvae and *Carcinus maenas* have intermediate levels of digestive enzymes and are able to transfer from herbivorous to omnivorous feeding. To date only decapod larvae which show high trypsin activity can be successfully reared to metamorphosis on formulated feeds. Inclusion of algal material, as a gut enzyme stimulant, for penaeid protozoeal stages and pre-digested ingredients for later stages into feeds are proposed.

CONTENTS

ACCOMPANYING MATERIAL

1. Kumlu, M. and Jones, D. A. 1993. Optimum rearing conditions for rearing *Penaeus indicus* larvae. European Aquacult. Soc. Spec. Pub. 19: p.142.

2. Kumlu, M., Le Vay, L. and Jones, D. A. 1994. Recent advances in the development of microencapsulated diets for shrimp larvae. In: Kas, H. S. and Hincal, A. A. (Eds.), Minutes of the 9th Int. Symp. On Microencapsulation Editions de Santé, Paris. pp. 205-208.

3. Fletcher, D. J., Fisher, C. M., Kumlu, M. and Rodgers, P. B. 1994. Growth and survival of *Penaeus indicus* larvae fed on the nematode *Panagrellus redivivus*. Proceedings of the World Aquaculture Society. New Orleans, Louisiana, USA. p.109.

4. 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, USA. p. 117.

5. Kumlu, M. and Jones, D. A. 1995. Role of microalgae as a gut enzyme stimulant in rearing *Penaeus indicus* larvae on microencapsulated diets. Book of Abstracts of the World Aquaculture '95' San Diego, February 1-4, 1995. World Aquaculture Society, Baton Rouge, Louisiana, USA. p. 158.

6. Kumlu, M. and Jones, D. A. 1995. Salinity tolerance of hatchery-reared postlarvae of *Penaeus indicus* H. Milne Edwards originating from India. Aquaculture, 130: 287-296.

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GENERAL INTRODUCTION

Some parts of this section have contributed to the following publication:

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GENERAL INTRODUCTION

While global shrimp landings from fisheries reached a plateau at approximately 1.9 million tons in 1985, farmed-shrimp production, particularly in Asia and South America, is still growing remarkably. The overall shrimp production increased from 0.7 million tons in 1965 to 2.5 million tons in 1990 and may exceed 3.2 million tons by year 2000 (Csawas, 1994). The Food and Agricultural Organization of the United Nations (FAO) reported that the volume of worldwide farmed-shrimp reached to 884,075 tons in 1992 (cited in Fish Farming, 1994) making up more than 30% of the global shrimp supply in 1992 (Csawas, 1994; Landesman, 1994). Nearly 80% of the total crustacean aquaculture production was in the form of marine shrimps in 1989 (New, 1991).

The practice of culturing shrimp was first developed in Japan with a native prawn, *Penaeus japonicus*, and then spread to other Asian countries e.g. Taiwan, Thailand, China, Indonesia and recently to South American countries such as Ecuador, Mexico, Colombia, etc. The eastern hemisphere produces 80 % of the farm-cultured shrimp and the western hemisphere 20 % (Weidner and Rosenberry, 1992). Shrimp industry provides one of the major sources of income in developing countries e.g. Ecuador, Bangladesh, Philippines (Landesman, 1994). Major cultured shrimp species are *P. monodon* (49.8 %), *P. chinensis* (13.8 %) and *P. vannamei* (15.5 %) (Csawas, 1994). *P. indicus*, which is cultured in extensive shrimp farms throughout South-East Asia, mainly in the Philippines, constitutes 5.4 % of the total farm-raised shrimp production in 1992 (Weidner and Rosenberry, 1992). Freshwater prawn aquaculture is in the interest of Asian countries mainly Thailand, Vietnam and Taiwan.

For many years, shrimp farms supplied their seed requirement from wild-caught postlarvae. However, wild seed stocks are limited and cannot meet

the demand of a fast growing industry that needs a continuous supply of postlarvae (PL) throughout the year. Hence, a vast number of hatcheries have been established to meet the demand for seed by the shrimp industry over the last few decades. It was estimated that there were 4,756 hatcheries in tropical countries in 1991 to supply seed for 36,840 shrimp farms (Weidner and Rosenberry, 1992). These hatcheries rely on wild collected gravid females for the production of shrimp nauplii. Hatchery management requires a proper water quality control and appropriate feeding regimes. In large and well-equipped hatcheries, sea water used in larval culture is generally filtered and UV-treated to prevent disease breakouts.

Live feeds

Penaeid larvae hatch as a non-feeding stage called a nauplius (ranges from 5-6 stages), and pass through three protozoeal (PZ1-3) stages and three mysis (M) stages before reaching the postlarval stage (PL). Penaeid hatcheries conventionally rear penaeid shrimp larvae on microalgae (diatoms, flagellates, etc.) during zoeal, and 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 (Sorgeloos et al., 1983; Langdon et al., 1985; Jones et al., 1993). Although *Artemia* is the most practical animal prey, limited resources, high cost of cysts, and nutritional variability are disadvantages of this live feed source (Sorgeloos, 1980; Watanabe et al., 1983; Léger et al., 1985, 1986). Use of mixed algal diets for penaeid shrimp larvae always gives superior survival, growth and development to single algal species (Kuban et al., 1985; Amjad, 1990) due to their more balanced nutrient content. While provision of live feeds is a general routine in most shrimp hatcheries, larval production on these feeding regimes may be inconsistent throughout the season (Cook and Murphy, 1969; Mock et al., 1980). Table 1 summarizes the success of using live feed to rear commercially important penaeid species during larval development.

Search for cheaper, nutritionally adequate and practical larval feed sources 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; Yúfera et al., 1984), but their use has been limited in hatcheries because of difficulties in mass culture and poor nutritional quality (i.e. highly unsaturated fatty acids = HUFA) (Watanabe et al., 1983). Attempts to replace *Artemia* with the rotifers in culture of a fresh water prawn species *M. rosenbergii* were unsuccessful (Lovett and Felder, 1988). Live free-living nematodes, *Panagrellus redivivus*, were suggested as a potential alternative live feed source to replace *Artemia* in the culture of several penaeid species (Wilkenfeld et al., 1984; Biedenbach et al., 1989). The nematodes can be cultured easily on cheap growth media in mass quantities and their nutritional value can be modified by loading their alimentary canal with growth factors such as HUFA (Kahan et al., 1980; Rouse et al., 1992).

Table 1. Microalgal and zooplankton feeds used to rear commercially important penaeid species.

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

Artificial diets

Different processing techniques have been employed to produce artificial particles in dehydrated forms as food for aquatic animals. All these processing methods were extensively reviewed by Langdon et al., (1985). Since then, spray drying techniques, which involve spraying a homogenized mixture of ingredients into hot air to form heat sealed and water-stable capsules, have increasingly been used to produce diets for penaeid larvae. Whichever processing method is used, the artificial diet must satisfy the same parameters: acceptability, digestibility, stability, adequate nutritional content, cost-effectiveness, and storage (Jones et al., 1993). After finding that artificial food particles are accepted by some filter feeding crustaceans (Jones et al., 1972), several kinds of artificial diets have been manufactured to replace the live feed, partially or totally. The most commonly used artificial diets to culture shrimp larvae are microbound (microparticulated) and microencapsulated diets (MED). Microbound diets are inexpensive, easy to produce and are reported to be used successfully in laboratory and hatcheries (Kanazawa et al., 1982; Galgani and Aquacop, 1988; Liao et al., 1988; Kanazawa, 1990). These diets are produced by mixing the nutritional ingredients thoroughly with binders (carboxymethyl cellulose, calcium alginate, carrageenan, agar or gelatine). The mixture is then oven- or freeze-dried, ground and finally sieved through appropriate sizes. They exhibit poor stability in water causing not only water pollution and bacterial built-up, but also they may become deficient due to nutrient leach loss (Amjad et al., 1992).

The microencapsulation technique was first modified from Chang et al., (1966) to deliver nutrients in a protein and nylon cross-linked membrane to prevent nutrient loss through leaching and used to identify specific nutritional requirements of aquatic organisms (Jones et al., 1979a, b). Further development of the technique resulted in the production of only cross-linked protein walled capsules, capable to withstand drying, which have

been used extensively in the laboratory and commercial hatcheries (Jones et al., 1987; Kurmaly et al., 1989a; Jones et al., 1993). Among a wide range of artificial diets manufactured in an attempt to completely or partially replace live diets in culture of penaeid larvae MED have proved to be the most successful (Jones et al., 1979a; 1984; 1987; Kurmaly et al., 1988; 1989a; 1989b; Amjad, 1990; Amjad and Jones, 1992; Jones et al., 1993). Although complete replacement of live diets with MED has been limited in success, partial replacement is already routinely used in many hatcheries (Jones et al., 1987; Fegan, 1992). These encapsulated diets promote good survival, but slower growth rate and development in penaeid shrimp and prawn larvae in comparison to live diets. Growth and survival equivalent to live diet has been reported for *P. monodon* (Amjad et al., 1992; Kamarudin, 1992; Jones et al., 1993) when a small amount of live or frozen algae (10 cells μl^{-1}) was used as a supplemental co-feed with microencapsulated diets. Recently, Ottogali (1991, 1993) reports successful results in complete replacement of algae in commercial hatcheries in culture of penaeid larvae. However, penaeid larval growth and development on live feeds are generally still superior to those solely on formulated diets (Galgani and Aquacop, 1988; Jones et al., 1993).

Complete replacement of live diets by artificial diets to rear caridean shrimp and homarid larvae is not currently possible. Live *Artemia* was replaced completely in *M. rosenbergii* culture, but only from stage Z6 to Z11 with a microencapsulated diet designed for penaeid larvae (Deru, 1990). Despite considerable efforts to develop an adequate artificial diet as a substitute and/or supplement (Brewster, 1987; Deru, 1990), hatchery production of *M. rosenbergii* still relies heavily on live *Artemia* at least during its early stages (Jones et al., 1993). The inability of the early larvae of these species to survive on artificial diets may be due to their feeding behavior and low digestive enzyme activities (Jones et al., 1993). Table 2

summarizes the success in larval rearing of crustaceans on formulated diets during larval development.

Table 2. Artificial diets used to replace live diets in culture of decapod crustacean larvae.

Species	Type of diets	Results	References
PENAEID			
P. indicus	Microbound diet	62 % survival to M1, growth inferior to algae	Galgani and Aquacop (1988)
P. japonicus	Microencapsulated diets	50% survival to postlarval stage	Jones, et al., (1979a)
P. japonicus	Microbound diet	90 % survival to PL1, growth less than live feeds	Kanazawa et al., (1982)
P. japonicus	Microencapsulated diets	90% survival to PL4, growth same as live feeds	Kanazawa (1985)
P. japonicus	Microbound diet	75 % survival to PL1	Kanazawa (1990)
P. japonicus	Microencapsulated diets / alga	79.5 % survival to PL1	Le Vay et al., (1993)
P. japonicus	Microencapsulated diets	43.8 % survival to PL1	Le Vay et al., (1993)
P. monodon	Microencapsulated diets	3-29% survival to PL7	Jones et al., (1987)
P. monodon	Microencapsulated diets /algae no *Artemia*	9-47 % survival to PL7	Jones et al., (1987)
P. monodon	Microencapsulated diets	80 % to PL1 growth inferior to live feed	Amjad et al., (1992)
P. monodon	Microencapsulated diets /algae (10 cells μl^{-1})	74 % to PL1 growth same as live feed	Amjad et al., (1992)
P. monodon	Microencapsulated diets	51-64% survival to PL	Kurmaly et al., (1989a)
P. monodon	Microbound diet	85 % survival to M1	Galgani and Aquacop (1988)
P. stylirostris	Microencapsulated diets /*Artemia*	Growth and survival equal to live feeds	Ottogali (1991)
P. stylirostris	Microencapsulated diets /algae	65% survival to PL5-7	Jones et al., (1987)
P. vannamei	Microencapsulated diets /algae / *Artemia*	90% survival to PL5 -7	Jones et al., (1987)
P. vannamei	Microencapsulated diets /algae without *Artemia*	80% survival to PL5 -7	Jones et al., (1987)
P. vannamei	Microbound diets	47% survival to M1	Galgani and Aquacop (1988)
CARIDEAN			
M. rosenbergii	Freeze dried catfish (from Z4 stage)	11% survival to metamorphosis	Sick and Beaty (1975)
M. rosenbergii	Nylon protein Microcapsules	Larvae survived to 4th stage	Jones et al., (1975)

Continued from Table 2

Species	Type of diets	Results	References
M. rosenbergii	Microencapsulated diets (from Z4 to PL stage)	84 % survival to PL1, slower growth than *Artemia*	Deru (1990)
Crangon nigri-cauda	*Artemia* - microcapsules	No survival beyond Z2 stage	Villamar and Brusca (1987)
LOBSTER			
H. gammarus	Microencapsulated and microbound diets	No survival beyond stage III	Kurmaly et al., (1990)
CRAB			
Eurypanopeus depressus	Microcapsules plus rotifers	83-93 % survival to megalopa	Levine and Sulkin (1984)
Portunus tritu-berculatus	Microcapsules plus rotifers	16.1 % survival to juvenile stage	Kanazawa et al., (1983)

Larval nutritional requirements

Various dietary requirements for different penaeid species have been studied. New (1976, 1980) provides an extensive bibliography on nutritional research for penaeids. Despite recent advances in understanding of nutrition of adult and juvenile decapod crustaceans (Kanazawa, 1984; 1990; Langdon et al., 1985; Liao and Liu, 1990; Guillaume, 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). Absolute nutritional requirement of penaeids can only be identified when a water stable formulated diet is accepted, ingested, digested and assimilated at comparable levels to live diets (Jones et al., 1993). Current larval artificial diets are manufactured using natural ingredients such as fish or shellfish meals, cod roe and other types and have similar nutritional value to that of live or zooplankton feeds. However, the percentage of the nutritional composition of microparticulated diets which reaches the larva varies as a result of species specific acceptability and stability of the diets.

From the nutritional value of live algae successfully used in penaeid culture, it may be concluded that penaeid larvae require a protein level of between 23-55 % of dry weight of diets (Liao and Liu, 1990; Akiyama et al., 1992; Rodríguez et al., 1994). It is generally accepted that penaeid larvae and postlarvae require higher dietary protein requirement than juvenile and adults (Kanazawa, 1984; 1990; Akiyama, 1992). However, Le Vay et al., (1993) recently demonstrated that *P. japonicus* larvae can be successfully reared to metamorphosis on the alga, *Chaetoceros gracilis*, which contain only 7 % protein (dry weight). Jones et al., (1979b) showed the importance of HUFA particularly 20: 5ω-3 and 22: 6ω-3 in *P. japonicus* larvae using nutritionally defined microcapsules. Lipid and carbohydrate levels used in formulated diets are in the range found in live algae and zooplankton. Current microencapsulated diets, which have been successfully used in penaeid culture, contain 52 % protein, 13-14 % carbohydrate, 12 % lipid and 2 % HUFA (Le Vay, 1994). Essential vitamins are generally included in artificial diets at levels higher than recommended (Kanazawa, 1990).

Feeding and Digestion

Larval development is associated with drastic change in digestive morphology and physiology particularly between zoeal (caridean, lobster and crab larvae), mysis (penaeids) and metamorphosis. An appropriate artificial diet for larval rearing can only be developed when the digestive morphology, physiology and feeding behavior of an organism are fully understood. Penaeid shrimp larvae obtain their food by filtering microalgae from the water at protozoeal stages, and capturing zooplankton at mysis and postlarval stages. Caridean larvae, however, consume zooplankton directly 24-36 h after hatching. Cell size of microalgae used to feed early shrimp larvae are generally between 5-20 μm in diameter, whereas the size range of animal prey ranges from 70 to 500 μm. *Artemia salina* nauplii are the only realistic live prey for both penaeid and caridean larvae such as *M. rosenbergii* until their early postlarval stages. Although there is much research on the nutri-

tional requirements of adults and juveniles (Yonge, 1924; Young, 1959; Dall, 1967; Andrews and Sick, 1972; Shewbart et al., 1973; Barker and Gibson, 1977; Kanazawa et al., 1981; 1982; Kanazawa, 1985), little is known about the feeding mechanism, digestion, digestive enzymes, assimilation, gut structure, and nutritional requirement of the larvae of decapods. Increasing demand for postlarvae by shrimp industry and the decreasing availability of postlarvae from the wild has encouraged investigators to concentrate on larval digestive physiology (Factor, 1981; Kanazawa et al., 1982; 1983; Sasaki et al., 1986; Jones et al., 1979a, b; 1984; 1989; Kurmaly, 1989; Abubakr, 1991; Lovett and Felder, 1990a, b; Abubakr and Jones, 1992; Le Vay et al., 1993).

Recent studies on the digestive system of penaeid and caridean shrimp larvae have contributed towards the understanding of the digestive physiology of these larvae. Mandibles of shrimp larvae are able to crush and masticate food particles before the ingestion. During planktonic stages, decapod crustacean larvae are chance encounter feeders and need a high density of food particles in suspension at all times. Once contact is made the chemical and mechanical cues become important, and the larvae either consume or reject the particles (Moller et al., 1979; Kurmaly et al., 1990). Penaeid shrimp larvae are less selective than caridean and homarid larvae, accepting inert particles even at mysis stages unless they contain noxious and toxic substances (Kurmaly et al., 1990).

The digestive system of penaeid shrimp larvae is very simple and lacks a gastric mill, and filter apparatus during herbivorous stages. Existence of the anterior midgut diverticulae (AMD) along with the small hepatopancreas (HP) has been described by Lovett and Felder (1989; 1990c) for *P. setiferus* and by Abubakr (1991), Abubakr and Jones (1992) for *P. japonicus*, *P. monodon*, *P. kerathurus* and *P. vannamei*. Digestion is conducted by enzymes, released mainly from the AMD rather than the HP during early lar-

val development (Abubakr and Jones, 1992). At mysis stages, the teeth of the gastric mill are fully developed and, thus, the larvae become increasingly carnivorous, retaining food longer and assimilating a higher percentage of energy from their prey (Jones et al., 1993). Although penaeid larvae exhibit low assimilation efficiency during herbivoral stages (Kurmaly et al., 1989a), their survival on microalgae and artificial diets is thought to be due to their short gastroevacuation time (GET) and a sufficient amount of digestive enzymes produced by the AMD (Abubakr, 1991; Abubakr and Jones, 1992; Jones et al., 1993).

Caridean shrimp larvae, such as *Palaemon elegans* and *M. rosenbergii*, however, lack the AMD and hence may have limited digestive capabilities during early stages due to an underdeveloped HP between Z1 and Z4-5 (Deru, 1990; Abubakr, 1991). A drastic increase in the HP volume observed in *P. elegans* (Abubakr, 1991) and *M. rosenbergii* (Deru, 1990) at Z4-5 stages and longer food retention time may increase their digestive capability. These carnivorous larvae appear to rely on prey autolysis for digestion especially during their early stages (Kamarudin et al., 1994). Lobster larvae (*Homarus gammarus*) display a high assimilation efficiency on live diets, but cannot reach metamorphosis (Kurmaly et al., 1990) on encapsulated diets due to their possibly low level of digestive enzyme secretion and long GET (Kurmaly et al., 1990; Abubakr, 1991; Jones et al., 1993).

Several authors suggest that exogenous enzymes from the prey may contribute to digestion process of fish or crustacean larvae with poorly developed guts (Lauff and Hofer, 1984; Munilla-Moran et al., 1990; Jones et al., 1993). Studies on digestive enzymes during larval development have contributed to a better understanding of digestive capability of the organism (Biesiot and Capuzzo, 1990; Harms et al., 1991; Kamarudin et al., 1994). This knowledge allows the design of formulated diets according to the re-

quirement of a particular species (Kamarudin, 1992). Although there are numerous investigations on digestive enzymes in adult decapod crustaceans (Gates and Travis, 1969; Van Weel, 1970; Hoyle, 1973; Gibson and Barker, 1979; Lee et al., 1984; Maugle et al., 1982; Glass et al., 1989), larval digestive enzymes have only recently been studied. Trypsin is the dominant proteolytic enzyme in decapod crustaceans and may be responsible from 40-60 % (Galgani et al., 1984; Tsai et al., 1986) of total protein digestion in penaeid larvae. These larvae show a high trypsin activity during protozoeal stages with a maximum level at M1-M2 stages, but the level declines through metamorphosis (MacDonald et al., 1989; Kamarudin, 1992; Kumlu et al., 1992; Jones et al., 1993; Le Vay et al., 1993). Table 3 shows larval digestive enzymes of decapod crustaceans studied to date.

It is generally thought that herbivorous decapods have high levels of carbohydrases but weak proteases whereas the reverse is true for carnivorous, while omnivorous ones are intermediate. Yonge (1937) found that carnivorous crustaceans have more active proteases and weak carbohydrases compared to herbivores. In contrast, Sather (1969) reported that omnivorous and herbivorous animals show higher proteolytic enzymes compared to carnivorous ones. Degkwitz (1957: cited in Sather, 1969), however, found no relation between digestive enzymes and feeding mode of crustaceans. Although there are several studies available in the literature for penaeid larvae (Galgani and Benyamin, 1985; MacDonald et al., 1989; Lovett and Felder, 1990a, b; Kamarudin, 1992; Kumlu et al., 1992; Le Vay et al., 1993; Rodríguez et al., 1994), caridean larvae (Van Wormhoudt, 1973; Kamarudin et al., 1994), lobster larvae (Biesiot and Capuzzo, 1990) and crab larvae (Harms et al., 1991; Harms et al., 1994), it is not possible to compare digestive capabilities of these species as these authors used different culture conditions, different assay methods, and expressed the enzyme activity in different units. In this study, International Unit (IU) is used to

express trypsin activity of larval decapods per larva or per µg dry weight (DW).

Table 3. Larval digestive enzymes in decapod crustaceans (+ refers present, - absent).

Species	Protease	Trypsin	Chitinase	Chymotrypsin	Carboxypeptidase A	Carboxypeptidase B	Elastase	Collogenase	Maltase	Lipase	Esterase	Amylase	Laminarinase	Reference
P. monodon	+	+	+	+					+	+	+	+		MacDonald et al., (1989); Kamarudin (1992); Kumlu et al., (1992); Fang and lee (1992)
P. japonicus	+	+									+	+		Laubier-Bonichon et al., (1977); Galgani and Benyamin (1985); Kamarudin (1992); Le Vay et al., (1993); Rodriguez et al., (1994)
P. setiferus	+	+		-	+	+				-	+	+		Lovett and Felder (1990a, b)
P. vannamei		+		+			+	+			+	+	+	Moullac et al., (1992)
M. rosenbergii		+									+	+		Kamarudin et al., (1994)
P. serratus	+											+		Van Wormhoud (1973)
H. americanus	+									+		+		Biesiot and Capuzzo (1990)
H. araneus		+										+		Hirche and Anger (1987); Harms et al., (1991)
C. maenas		+										+		Harms et al., (1994)

Aims of the present studies

• Chapter 1 investigates optimal culture conditions for *P. indicus* larvae fed locally available various algal species in order to establish a control treatment prior to further nutritional studies with artificial diets. Three algal species are compared at different cell densities and combinations on larval survival, growth and development of *P. indicus*. Optimal salinity during larval culture is also established.

• Chapter 2 investigates postlarval salinity tolerance of *P. indicus* originating from India to determine whether this population shows different salinity preferences to *P. indicus* cultured in the Red Sea (Bukhari et al., 1994). Optimal culture salinity between (postlarvae 7) PL7 and PL60 is established.

• Chapter 3 investigates the use of free-living nematodes as an alternative live feed source in the culture of two caridean species, a native prawn *P. elegans* and a freshwater prawn *M. rosenbergii*, and a penaeid species, *P. indicus*, during larval development. Complete replacement of live algae and

Artemia in the culture of *P. indicus* is demonstrated from PZ1 to PL stages.

- Chapter 4 aims to improve nutritional value of the nematode *P. redivivus* in an attempt to obtain larval (*P. indicus*) growth and development on the nematodes comparable to that on live algae and *Artemia*. The effects of astaxanthin and lipid-enriched nematodes are investigated on larval survival, growth, development and pigmentation. The effects of different algal co-feeds in addition to nematodes are examined on larval performance and trypsin activity.
- Chapter 5 examines the influence of live and artificial diets (MED) on growth, survival, development and trypsin activity of *P. indicus* larvae. Total replacement of live algae/*Artemia*, and the effect of algae included MED on larval trypsin activity are also investigated
- Chapter 6 investigates feeding behavior and digestive capability of two caridean species, *P. elegans* and *M. rosenbergii*, during larval development. The effects of live and artificial diets on growth, survival, gastroevacuation time and trypsin activity are also examined.
- Chapter 7 compares the specific trypsin activity of several decapod crustacean species and discusses the possibility of using specific trypsin activity (IU μg^{-1} larval dry weight) as a tool to describe feeding strategy of decapod larvae. Larval trypsin activity of *H. gammarus*, *N. norvegicus* and *C. maenas* are also examined.

GENERAL MATERIALS AND METHODS

Maturation facilities

Penaeus indicus broodstock originating from India and reared in Tahiti and France, IFREMER for several generations was kept in a black circular plastic tank (capacity 6 m^3; diameter 2.80; height 1.10 m) situated in a tropical conditioned room. The bottom area of the tank was 6.12 m^2. The temperature of the room with insulated walls was maintained at 35 °C by fan heaters. A controlled photoperiod (10L:14D) with artificial fluorescent illumination was applied and water temperature was maintained at 28 ± 0.5 °C by thermostatistically controlled heaters. The water level of the tank was kept low (0.60 m) to observe maturation stages of females easily. The water of the tank was recirculated through two bio-filters connected to the tank by a pump (max. flow: 50 l min^{-1}). Each bio-filter consisted of 2 circular transparent plastic columns (diameter 47 cm; height 2 m) filled with plastic artificial media to act as a substrate for nitro-bacteria. The sea water pumped on top of these bio-filters fell by gravity over these artificial media. Air supplied by a blower diffused through a large air stone. Fresh sea water was dripped continuously into the tank to change 1/3 of the water of the tank daily. Every morning, wastes, moults and dirt were siphoned out from the bottom of the tank. The pH, nitrate, nitrite, ammonia contents of the water were checked by using Tetra Test kits. Initially 12 males and 6 females, of which some features are summarised in Table 1, were stocked in the tank. Following any mortality, the dead animals were replaced.

Table 1. Mean weight (g), carapace length (mm), and total length (mm) of *Penaeus indicus* broodstock.

	Males	Females
Mean weight (g)	10.48 ± 1.15	15.15 ± 3.01
Carapace length (mm)	3.97 ± 0.23	4.60 ± 0.30
Total length (mm)	10.19 ± 0.41	11.25 ± 0.49

Treatment of the animals

Different ablation techniques, such as making an incision across the eyeball and squeezing the contents outwards, pinching the eyestalk, cutting the eyestalk, were tested. After a few trials, it was concluded that cutting the eyestalk, after it had been tied with a piece of string, results in much better success in lowering mortality of females. Therefore, this ablation technique was applied commonly for *P. indicus* females. Males did not need any ablation. No application of antibiotics was necessary after the ablation. The broodstock animals were fed predominantly on fresh mussel, *Mytilus edulis*, lugworm (*Arenicola marina*), and chopped squid in excess. Mussels were opened, cleaned and given with their shells everyday. The remaining of the food was siphoned out everyday or sometimes twice a day.

Description of the hatchery procedures

P. indicus females were normally staged on a daily basis. Ovaries were checked during late afternoon by a flash light to outline their stage of maturation. Females ready to spawn was removed to a black 100-l spawning tank supplied with a heater and gentle aeration (28 °C and 33.5 ppt). The tank was left undisturbed until the next morning. The female (spawned or not) was returned to the maturation-mating tank.

Spawning Treatments

Viability of the eggs was evaluated under a light microscope and their number was estimated. After hatching, 50% of the water was renewed with filtered (0.2 µm) and UV-irradiated sea water. Once the larvae developed through the sixth nauplius (N6) stages to protozoea 1 (PZ1), a 80 µm mesh was used to lower the water of the spawning tank to 10 cm water depth. Remaining water was siphoned through 350 µm mesh to eliminate large faecal matter and other wastes. The larvae were rinsed in the filtered and UV-treated sea water and generally treated with an antibiotic (furazolidone) at 0.2 ppm before being used in feeding experiments.

Trypsin Analysis

Whole homogenates were used in the enzyme assays; the larvae were homogenised in a glass tissue grinder with tris buffer (46 mM: pH = 8.1; 11.5 mM $CaCl_2$), which was prepared according to the method of Rick (1974). The homogenised samples were centrifuged for 5 min at 12000 rpm at room temperature. Two replicates for each treatment and triplicate assays for each sample were conducted and used for calculations. Trypsin-like enzyme activity was assayed by using 10 mM Nα-p- Toluenosulfonyl- L-Arginine Methyl Ester (TAME) at pH 8.1, and 25 °C (Rick, 1974). Different amounts of substrate and buffer solutions, depending on the stage of the larvae, were equilibrated at 25 °C in 1.5 ml micro test tubes. Following the addition of the sample, the mixture was transferred to a 1 cm path length quartz cuvette, placed in a thermostatically controlled holder at 25 °C. Changes in absorbance at 247 nm were measured every 6 s for 180 s by a Hewlett Packard 8452A Diode Array spectrophotometer. Trypsin activity in the sample was calculated as follow:

$$\text{Total trypsin activity} = \frac{\text{Sample vol. x Assay vol.}}{\text{Sample vol. x } \in \text{ x d in assay}} \times \delta Abs/\delta t$$

$\in$ = Extinction coefficient (0.54 $\mu mole^{-1}$ for TAME)
Abs = Absorbance,
t = time (min)
d = Path length (cm)

Total enzyme level is divided by the number of the larvae to give total trypsin activity in International Unit (IU) $larva^{-1}$. Specific trypsin activity (IU μg^{-1} dry weight) is found by dividing the enzyme level to individual larval body dry weight (DW). One IU is equivalent to one µmole of substrate hydrolysed in one minute.

Determination of larval body dry weight

Larval samples were washed in distilled water and dried on pre-weighed fine meshes in an oven at 60 °C for 24-36 h. After cooling in a desiccator for 1-1.5 h, the samples (two replicates for each stage) were weighed on a microbalance (CAHN-31).

CHAPTER 1

OPTIMAL LARVAL CULTURE CONDITIONS FOR *P. indicus* (FROM INDIA).

Parts of this chapter were presented at 'The International Conference WORLD AQUACULTURE '93, Torremolinos, Spain, May 26-28, 1993.

Title : Optimum rearing conditions for *P. indicus* larvae reared in the laboratory.

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INTRODUCTION

It has been possible to close the life cycle in captivity for *Penaeus indicus* with stock originating from India now bred through several generations in partial recirculation systems in the School of Ocean Sciences, Gwynedd Menai Bridge, UK. This disease free stock presents an ideal opportunity for larval nutritional research and genetic studies as it is possible to produce disease free larvae at regular intervals throughout the year. Present work describes the optimisation of larval culture for this disease free stock using live feeds.

Penaeid larvae are generally cultured on live unicellular algae during protozoeal stages and animal prey are added along with the algal feeds during mysis and early postlarval stages (Hudinaga, 1942; Cook and Murphy, 1969). Several species of algae such as the phytoflagellates *Tetraselmis* sp. (Hudinaga, 1942; Samocha and Lewinsohn, 1977; Kurmaly et al., 1989a) and *Isochrysis* sp. (Aquacop, 1984), diatoms *Skeletonema* sp. (Yúfera et al., 1984; Preston, 1985b), *Thalassiosira* sp. (Emmerson, 1984; Kuban et al., 1985) and *Chaetoceros* sp. (Tobias-Quinitio and Villegas, 1982; Aquacop, 1984; Kuban et al., 1985) have been reported to be adequate as food for penaeid larvae. Some authors such as Griffith et al., (1973) state that phytoflagellates sustain better larval growth and survival than the diatoms, while others such as Tobias-Quinitio and Villegas, (1982); Aujero et al., (1983) report that diatoms are better live feeds for rearing penaeid larvae. These contradictory results were attributed to cell size and different nutritional contents of microalgal species (Aujero et al., 1983). Hence, penaeid larvae are generally cultured on mixed algal feeds to eliminate poor survival and growth rates resulting from possible nutritional deficiencies in single algal species. While some penaeid species such as *P. vannamei* were grown on mixed diatom algal species of S*keletonema* / *Chaetoceros* successfully (Kuban et al., 1985), others such as *P. monodon* larvae were cul-

tured on mixed flagellate species of *Tetraselmis chuii/ Rhinomonas reticulata* (Kurmaly et al., 1989a). Of the live animal prey used as food during mysis and PL stages in penaeid larviculture, freshly hatched *Artemia* nauplii are the most widely used species. In some cases other live zooplankters such as the rotifer *Brachionus plicatilis* (Emmerson, 1984; Yúfera et al., 1984) and the nematode *Panagrellus redivivus* (Samocha and Lewinsohn, 1977; Wilkenfeld et al., 1984) are used as live feeds to culture penaeid larvae during mysis stages. A number of investigations have concentrated on finding the best algal food sources and the larval stage at which animal prey should be offered to penaeid larvae (Gopalakrishnan, 1976; Kuban et al., 1985).

Larval cultures of *Penaeus indicus* have received considerably less attention compared to other commercially important penaeid species. Larvae of this species have been cultured successfully on various single algal species, such as *Thalassiosira weissflogii* (Emmerson, 1980), and on mixed algal diets, such as *Chaetoceros gracilis*, *Platymonas* sp., and *Isochrysis aff. galbana*, and *Artemia salina* nauplii after PZ3 stage (Galgani and Aquacop, 1988). Aquacop (1983) suggests the use of mixed algae *Isochrysis* and *Chaetoceros* in rearing *P. indicus* larvae. The optimal cell concentration for growth and survival of penaeid larvae varies with larval developmental stages and cell size of algal species used. Emmerson (1980) obtained 96 % survival to PL1 when he maintained algal cell density of *T. weissflogii* at 7 cells μl^{-1} between PZ1 and PZ3 stages. Aquacop (1983) recommends 100 cells μl^{-1} of the mixed algae of *Chaetoceros* (20 %) and *Isochrysis* (80 %) between PZ1 and PZ3 stages. Galgani and Aquacop (1988) report that algal cell density of 30-40 cells μl^{-1} of *C. gracilis*, *Platymonas* sp., and *Isochrysis aff. galbana* was adequate to rear *P. indicus* larvae during protozoeal stages. Emmerson and Andrews (1981) studied the effect of stocking density on the growth, survival and development of *P. indicus* and concluded that levels decrease with increasing larval stocking density.

Preliminary experiments with *P. indicus* larvae obtained at the School of Ocean Sciences, Menai Bridge, UK, have shown that mixed algae (*T. chuii*/ *R. reticulata*) which has been used successfully to rear *P. monodon* (Kurmaly et al., 1989a; Amjad, 1990, Kumlu et al., 1992) at cell density of 45-50 cells μl^{-1} is not suitable for culturing *P. indicus*. Hence, in the present work, evaluation of live diets was conducted to determine the best diet to promote high survival and rapid growth for this larval species so that it can be used as a control diet for further nutritional studies with this penaeid. In the first experiment, *Tetraselmis chuii*, *Rhinomonas reticulata* and *Skeletonema costatum* were tested singly and in combinations from 10 to 50 cells μl^{-1} from PZ1 to PL1 stage. In the second experiment, the best algal feeds were selected and algal cell concentration was increased from 50 to 80 cells μl^{-1} as the first experiment showed that optimal algal cell concentration was in the higher range. The third experiment investigated the possibility of eliminating algae totally during the culture of mysis stages. A fourth experiment was conducted to assess the effect of four salinities (from 20 to 35 ppt) on larval survival and growth of *P. indicus*. In all experiments larvae were fed on algal feeds (protozoeal stages) plus five *Artemia* nauplii ml^{-1} after stage PZ3/M1 (mysis and postlarval stages). The fifth experiment investigated possible reasons as to why *R. reticulata* is not a suitable live feed alone or in combination with other algae as food for *P. indicus* larvae. The primary aim of this study was simply to ascertain the best of the available algal species and cell concentrations together with optimum environmental conditions that promote maximum larval growth and survival in *P. indicus*.

MATERIAL AND METHODS

Experiment 1

P. indicus larvae were obtained from broodstock, originating from India, kept in the School of Ocean Sciences (see General Material and Methods).

Gravid females were spawned in 100-l tanks in filtered (0.2 μm) and U/V irradiated sea water. Following the non-feeding nauplius stages, PZ1 (protozoea 1) larvae were stocked in filtered and U/V treated sea water at 33.5 ppt salinity in 2-litre round bottom glass flasks in a water bath at 28 °C. Live monospecific algal cultures of three species, *T. chuii* (Butcher), *S. costatum* (Greville) and *R. reticulata* (Lucas), grown in a semi-continuous culture as described by Walne (1966), were fed to *P. indicus* larvae singly and in combinations (50 % from each algal species) at cell densities of between 10, 20, 30, 40 and 50 cells μl^{-1} day^{-1}. Algal diets used in the experiment were;

(1) *T. chuii,*

(2) *S. costatum,*

(3) *R. reticulata,*

(4) *T. chuii / S. costatum* (1:1),

(5) *T. chuii / R. reticulata* (1:1),

(6) *R. reticulata / S. costatum* (1:1).

A portable laboratory Quantum Scalar Irradiance Meter (Model QSL-100) was used to measure the light intensity of the culture room where all the following experiments in this thesis were conducted. Algal cultures were maintained in the exponential growth phase and Conway Medium was used as a source of nutrients (Walne, 1966). Everyday algal cell densities were estimated using a haemocytometer and a Coulter Counter (Model ZB: Coulter Electronics) both in the algal culture medium and larval culture flasks to maintain desired experimental algal cell densities. Tables 1 and 2 give description and nutritional contents of the algal species used in the present experiments. Complete water changes were carried out every other day when 10-15 larvae were measured under a binocular microscope from tip of the rostrum to the end of the tail (total length = TL), counted using glass pipettes and staged according to Silas et al., (1978). Five newly hatched (at 28 °C and 34 ppt for 24 h) *Artemia* nauplii (INVE AQUACUL-

TURE, Belgium) ml^{-1} were fed to the larvae along with the algal feeds from PZ3/M1 onwards. Larval growth and survival were assessed from two replicates from PZ1 to PL stages.

Table 1. Microalgal species and their nutritional contents (obtained from Kurmaly et al., 1989a) used in the present experiments.

Species	Cell size (µm)	Description	Protein (%)	Carbohydrate (%)	Lipid (%)	Ash (%)
Tetraselmis chuii	10-15	Flagellate	48.80	24.70	4.3	22.2
Skeletonema costatum	8-10	Diatom	33.30	22.60	8.1	36.0
Rhinomonas reticulata	8-10	Flagellate	52.00	33.70	4.3	4.3

Experiment 2

The first experiment showed that optimal cell density for *P. indicus* was higher than the range (10-50 cells μl^{-1}) tested. Hence, higher cell densities of 50, 60, 70 and 80 μl^{-1} day^{-1} of mixed algae *T. chuii / S. costatum* and *S. costatum* singly were tested on growth and survival in this experiment. Cell density of *T. chuii* was kept constant at 25 cells μl^{-1} in the mixed diets whilst cell density of *S. costatum* was changed between 25 to 55 cells μl^{-1}. All other experimental procedures were identical to the first experiment.

Experiment 3

The effect of algae, along with live *Artemia,* on growth and survival of mysis stages of *P. indicus* was investigated in this experiment. For this purpose the PZ3/M1 larvae, previously reared on live mixed algae (25 cells μl^{-1} *T. chuii* and 35 cells μl^{-1} *S. Costatum*), were stocked in 2-l experimental round bottom glass flasks at a density of 75 larvae l^{-1}. Larvae were fed on three feeding regimes of mixed algae, mixed algae plus five *Artemia* ml^{-1}, and five *Artemia* ml^{-1} only from PZ3/M1 to PL stages. Algal cell density

was increased from 60 cells μl^{-1} (Z3/M1) to 70 (M2/M3) and finally 80 cells μl^{-1} (PL1) by increasing the cell density of *S. costatum* to ensure that there was sufficient amount of food in the culture medium at all time without causing larval fouling. Complete water changes of the flasks were performed everyday when staging, growth and survival measurements were performed from two replicates for each feeding regime. Rearing water was maintained at 25 ppt salinity (S), which was found to be optimal during larval culture of *P. indicus* (see Experiment 4), by mixing the filtered and UV-treated sea water with distilled water.

Experiment 4

The effect of salinity (20, 25, 30 and 35 ppt) on growth and survival of *P. indicus* was investigated from PZ1 to PL stages in this experiment. Distilled water was used to reduce salinity of local sea water (filtered to 0.2 µm and U/V irradiated) to test salinities of 20, 25 and 30 ppt. Aquarium salt "Instant Ocean" (Aquarium systems) was added into the local sea water (33.5 ppt) to obtain 35 ppt saline water. Survival and growth were assessed from two replicates every other day. Larvae were stocked at a density of 100 individuals l^{-1} and fed on mixed algae (25 cells μl^{-1} of *T. chuii* and 35 cells μl^{-1} of *S. costatum*) and five *Artemia* ml^{-1} from PZ3/M1 onwards.

Experiment 5

This experiment was conducted to test again the suitability of the red algae *R. reticulata* as food for *P. indicus* larvae. Growth and survival of the larvae were assessed from two replicates in 2-l flasks on *R. reticulata* at 50 cells μl^{-1} and mixed algae *T. chuii /S. costatum* as control at 60 cells μl^{-1} from PZ1 to PZ3/M1 stages. PZ1 stage larvae were also starved in two flasks to determine how long they could survive without food. Two 2-l flasks were also set up without larvae to determine settlement rate of the cells of *R. reticulata* (50 cells μl^{-1}) over a 24 h period. Algal cell concen-

tration was determined using a Coulter Counter (Model ZB) and a haemocytometer respectively. Larval rearing sea water was maintained at 28 °C and 25 ppt by mixing filtered and UV-irradiated sea water with distilled water. Culture water was exchanged completely every day when the larvae were measured for total length and counted. Larvae were observed continuously to ascertain whether they were ingesting and digesting the algae by examining the gut and faeces under a microscope at each protozoeal stages.

Statistical analysis

In the first and second preliminary experiments, since survival and growth data did not fit in the general linear model (GLM in Minitab), larval survival and growth by algal feeds at various algal cell concentrations (10-50 cells μl^{-1} and 50-80 cells μl^{-1}) were analysed by two-way analysis of variance (two-way ANOVA) together with one-way ANOVA separately for algal species and cell concentrations at PZ3/M1 and PL1 stages. Appropriate multiple pairwise comparison tests (Tukey's for equal sample sizes and Scheffé's method for unequal sample sizes) were performed to determine any significant effects ($P \leq 0.05$) of the treatments on larval growth and survival. Before the statistical analysis, data was checked for normality and homogeneity of variances using Bartlett's Box test (Sokal and Rholf, 1981) using Minitab. Linear regression lines of the data from third, fourth and fifth experiments were compared by the method of two-way ANOVA with days as a covariate, after linearity of the data was examined by residual plots from regression analysis, to determine any significant effect of treatments on larval growth rates and survival rates between PZ1 and PL1 stages. Increase in total length (growth rate mm day^{-1}) and decrease in survival (slope of each treatment), which was expressed as mortality rates (% day^{-1}), were derived from the output of the analysis and given in each ANOVA table. All statistical analyses were conducted using the facilities in Minitab statistical software.

Table 2. Percentage fatty acid composition of microalgae used in the present experiments to feed *P. indicus* larvae. (Data obtained from Kurmaly et al., 1989a).

	Flagellates		Diatom
	T. chuii	*R. reticulata*	*S. costatum*
Saturates			
12:0	-	-	-
14:0	4.0	4.9	19.7
15:0	-	-	0.5
16:0	26.0	11.0	10.7
17:0	-	-	-
18:0	-	-	-
20:0	-	-	-
22:0	-	-	-
24:0	-	-	-
sum %	30.0	15.9	30.9
Monounsaturates			
16: 1 (ω-10)			-
16: 1 (ω-9)	0.7	0.5	-
16: 1 (ω-7)	0.6	1.3	32.6
16: 1 (ω-5)	-	-	-
16: 1 (ω-13) t	-	-	-
18: 1 (ω-10)	-	-	-
18: 1 (ω-9)	11.5	3.9	1.7
18: 1 (ω-7)	4.3	3.2	1.2
20: 1 (ω-9)	-	-	-
sum %	17.1	8.9	35.5
Polyunsaturates	-	-	
16: 2 (ω-7)	-	-	-
16: 2 (ω-6)	-	-	-
16: 2 (ω-4)	-	-	-
16: 3 (ω-6)	-	-	-
16: 3 (ω-4)	-	-	-
16: 3 (ω-3)	-	-	-
16: 4 (ω-3)	-	-	-
16: 4 (ω-1)	-	-	-
18: 2 (ω-9)	-	-	-
18: 2 (ω-6)	11.5	14.2	1.1
18: 3 (ω-6)	2.6	2.7	0.2
18: 3 (ω-3)	23.1	16.5	0.9
18: 4 (ω-3)	9.5	17.3	4.4
20: 1 (ω-9)	0.7	0.4	-
20: 4 (ω-6)	0.4	3.0	0.2
20: 4 (ω-3)	-	-	-
20: 5 (ω-3)	3.4	11.6	22.9
22: 1 (ω-11)	-	0.2	-
22: 5 (ω-3)	-	0.1	-
22: 5 (ω-6)	-	-	-
22: 6 (ω-3)	-	7.6	2.9
Sum %	51.2	73.6	32.6
Others	1.7	1.6	1.0
Total	100	100	100

RESULTS

Experiment 1

(a) Larval growth and survival (PZ1-PZ3/M1)

Survival and growth of *P. indicus* larvae fed various algal feeds at different cell densities (10-50 cells μl^{-1}) are given in Tables 3a and 3b. Since cell densities of 10 and 20 cells μl^{-1} did not support survival and growth to PZ3/M1 stages, only the algal feeds at cell densities of between 30 and 50 cells μl^{-1} were compared statistically by two-way ANOVA. Figures 1a, b and Tables 5a and 9a show that there were significant effects of algal feeds and cell densities (30-50 cells μl^{-1}) on larval survival and growth at PZ3/ M1 stages ($P<0.001$). Highest survival (63 %) and best growth (3.72 mm TL) were achieved with the mixed algae (*T. chuii* / *S. costatum*, 1:1) at $P<0.05$ (Table 5b and 9b). Larval growth obtained from the flagellate, *T. chuii* (2.71 mm TL), was significantly inferior to that obtained from the diatom, *S. costatum* (3.26 mm TL) (Table 9b).

Multiple pairwise comparison tests (Tables 5b, c and Tables 9b, c) show that higher growth and survival were supported as algal cell concentrations increased from 30 to 50 cells μl^{-1}. Highest growth and survival were obtained when the larvae were fed at 50 cells μl^{-1} whereas the lowest growth and survival were supported by 30 cells μl^{-1}. Since there were significant interactions between algal feeds and cell concentrations on larval growth and survival (Tables 5a and 9a), further one-way analysis of variances were performed to determine the effects of algal diet and cell concentrations independently on larval survival (Tables 6-8) and growth (Tables 10-12).

10 cells μl^{-1}

Regardless of the species of algae tested, 10 cells μl^{-1} of single or even mixed algal diet did not promote survival further than stage PZ1. In all

cases larval mortality was total by the third day of the experiment (see Tables 3a, b).

20 cells μl^{-1}

Larvae fed either *T. chuii* or *S. costatum* survived until the 4th day of the experiment developing into PZ2 stage whereas *R. reticulata* fed larvae died on day 2 without moulting into PZ2 stage. Among the mixed algal feeds only *T. chuii / S. costatum* supported larval growth (2.58 mm TL) and survival (28 %) until PZ3/M1 stage. Other mixed algal diets did not support growth and survival further than PZ1 stage at this algal cell concentration (Tables 3a, b).

30 cells μl^{-1}

Among single algal feeds, *T. chuii* and *S. costatum* supported larval growth and survival until PZ3/M1, whereas *R. reticulata* fed larvae died on day 2. This alga in combination with *T. chuii* and/or *S. costatum* did not promote survival and growth beyond day 2. As shown in Figures 1a, b significant differences ($P<0.001$) were found between algal feeds at 30 cells μl^{-1} using one-way ANOVA on larval survival and growth at PZ3/M1 stages (Tables 6a, 10a). Multiple pairwise comparison tests (Tables 6b, 10b) show that mixed algae (*T. chuii / S. costatum*) gave significantly better survival (50.5 %) and growth (3.14 mm TL) than *S. costatum* (14%, 2.59 mm TL) and *T. chuii* singly (15%, 2.54 mm TL) at this cell density. There was no significant difference between growth and survival of larvae fed on the single algal feeds ($P>0.05$).

40 cells μl^{-1}

Among algal species, only *R. reticulata* did not support larval survival and growth of *P. indicus* at 40 cells μl^{-1} beyond PZ1 stage. Combination of this alga with *S. costatum* (1:1) promoted 19.73% survival until day 4, but only one larva developed into PZ3 stage at this cell density. One-way analysis of variance shows significant differences in larval growth ($P<0.001$) and sur-

vival at this cell density ($P<0.05$) (see Figures 1a, b and Tables 7a and 11a). Highest larval growth and survival at PZ3/M1 stages were attained on the mixed algae (3.69 mm TL, 59%) whereas the lowest were obtained from those fed on *T. chuii* singly (2.71 mm TL, 39%). There was no significant difference ($P>0.05$) between larval survival promoted by *S. costatum* (40 %) and *T. chuii* (39 %) at PZ3/M1 stages (Table 7b). However, larval lengths were significantly ($P<0.05$) greater on the diatom (3.34 mm) than the flagellate (2.71 mm TL) (Table 11b).

50 cells μl^{-1}

Highest larval survival (80 %) (see Figure 1a and Tables 8a, b) and growth (4.08 mm TL) (Figure 1b and Tables 12a, b) were again supported by the mixed algal feed (*T. chuii* / *S. costatum*) at this highest cell concentration tested in the present experiment. Although larval survival was higher on *S. costatum*, no significant difference was found between larval survivals supported by the two single algal species ($P>0.05$). Total length of larvae fed *S. costatum*, however, was significantly greater (3.92 mm) than those fed *T. chuii* (3.59 mm). When *P. indicus* larvae were fed *R. reticulata* at a cell density of 50 cells μl^{-1}, 23 % of the larvae developed into PZ2/PZ3 stages, but never reached M1 stage. This algal species in combination with *S. costatum* supported 10.5 % survival until PZ3/M1 stages (Tables 3a, b). *T. chuii* / *R. reticulata* mixed algae fed larvae died on day 4 (PZ2 stage).

Figure 1a

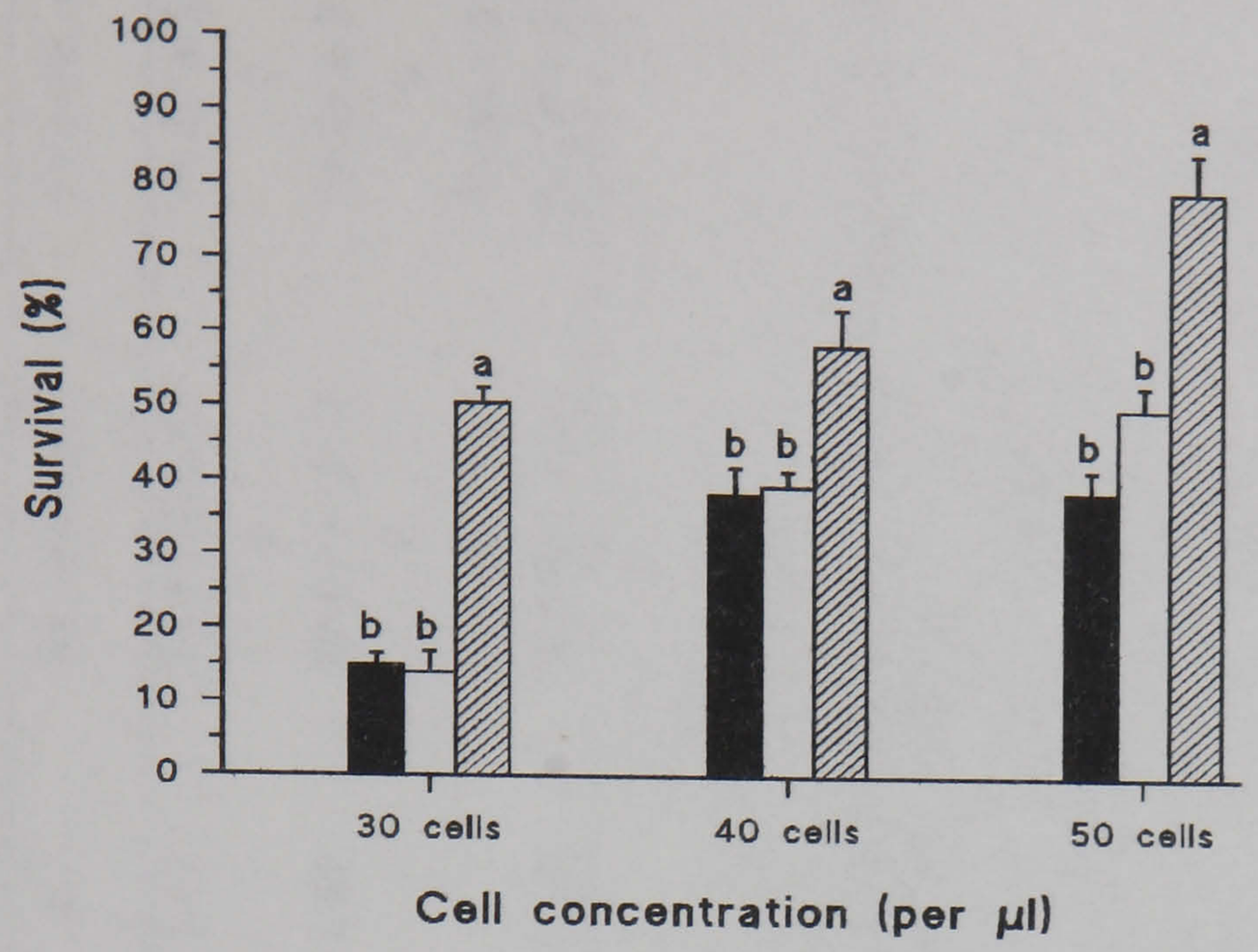
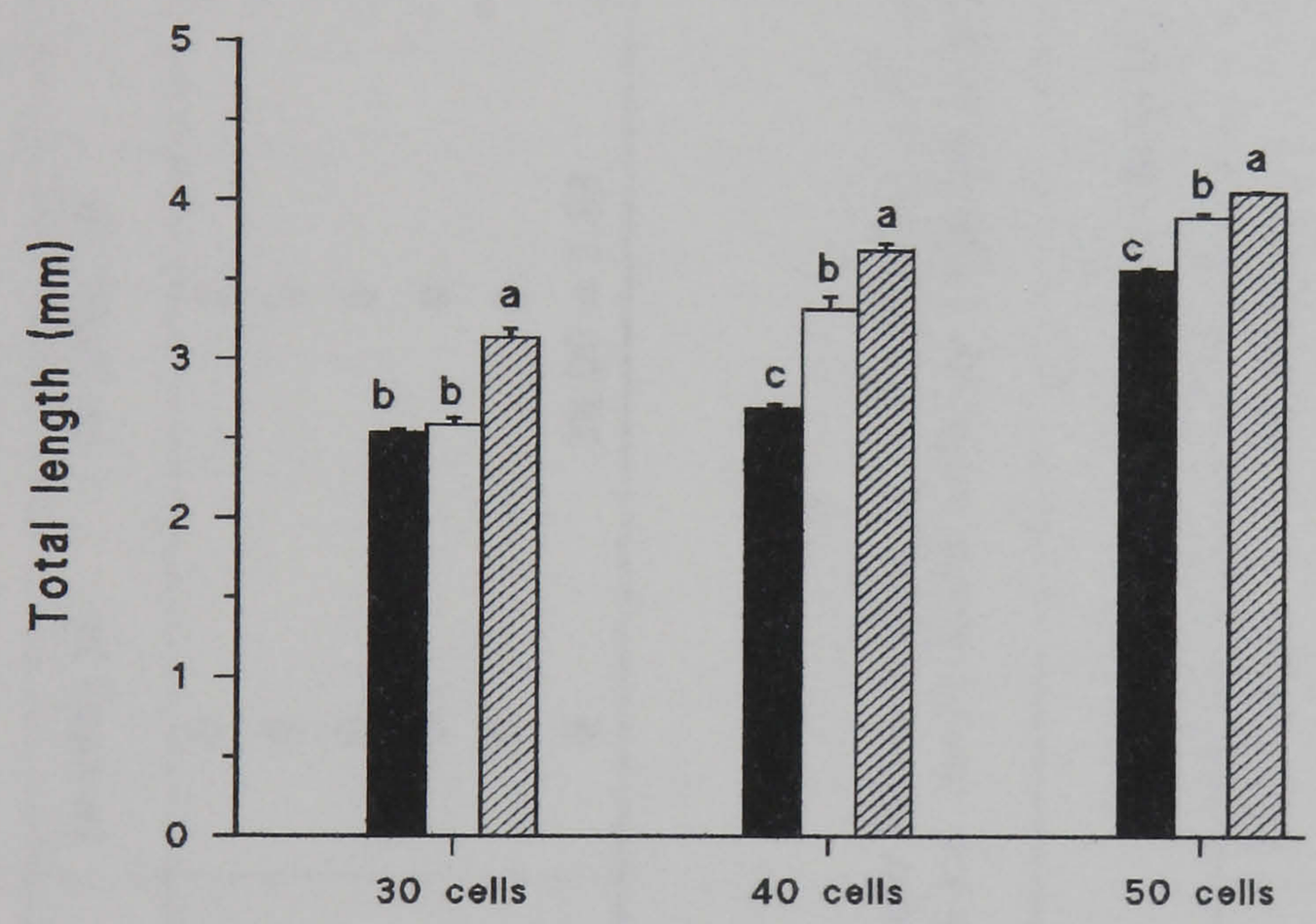


Figure 1b



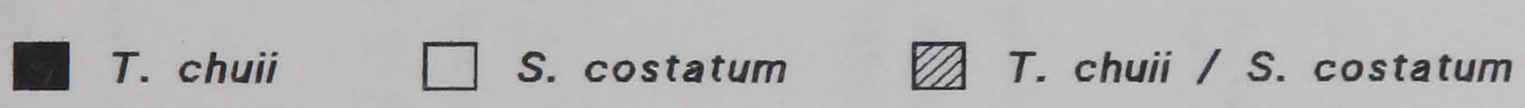


Figure 1. (a) Larval survival (%) and (b) growth (mm TL) of *P. indicus* reared on three algal combinations (*T. chuii*, *S. costatum* and *T. chuii* / *S. costatum*) at five algal cell concentrations of 10, 20, 30, 40 and 50 cells μl^{-1} from PZ1 to PZ3/M1 stages. Since 10 and 20 cells μl^{-1} did not promote survival until PZ3/M1 stages, they are not included in the figure. Each bar represents a mean ± s.d. (n=2). Each replicate contains measurements of 10-13 larvae for growth. Treatments marked with different superscripts are significantly different ($P<0.05$).

Table 3a. Survival (%) of *P. indicus* larvae reared on algal feeds at cell concentrations of between 10 and 50 cells μl^{-1} from PZ1 to PZ3/M1 stages. Each value is a mean ± s.d. (n=2).

	Larval survival (%) ± s.d. (PZ3/M1)				
Algal species/Cell concentration	10 cells μl^{-1}	20 cells μl^{-1}	30 cells μl^{-1}	40 cells μl^{-1}	50 cells μl^{-1}
T. chuii	0	0	15.00 ± 1.41	38.50 ± 3.54	39.00 ± 2.83
R. reticulata	0	0	0	0	0
S. costatum	0	0	14.00 ± 2.83	39.50 ± 2.12	50.25 ± 3.18
T. chuii / R. reticulata (1:1)	0	0	0	0	0
S. costatum / R. reticulata (1:1)	0	0	0	0	10.50 ± 3.54
T. chuii / S. costatum (1:1)	0	28.00 ± 2.83	50.50 ± 2.12	58.50 ± 4.95	79.75 ± 5.30

Table 3b. Total length (mm) of *P. indicus* larvae reared on algal feeds at cell concentrations of between 10 and 50 cells μl^{-1} from PZ1 to PZ3/M1 stages. Each value is a mean ± s.d. (n=2). Each replicate contains measurement of 10-13 larvae.

	Larval growth (mm) ± s.d. (PZ3/M1)				
Algal species/Cell concentration	10 cells μl^{-1}	20 cells μl^{-1}	30 cells μl^{-1}	40 cells μl^{-1}	50 cells μl^{-1}
T. chuii	0	0	2.54 ± 0.02	2.71 ± 0.02	3.59 ± 0.01
R. reticulata	0	0	0	0	0
S. costatum	0	0	2.59 ± 0.04	3.33 ± 0.08	3.92 ± 0.03
T. chuii / R. reticulata (1:1)	0	0	0	0	0
S. costatum / R. reticulata (1:1)	0	0	0	0	2.85 ± 0.07
T. chuii / S. costatum (1:1)	0	2.58 ± 0.08	3.14 ± 0.06	3.70 ± 0.05	4.08 ± 0.01

(b) Larval growth and survival (M1-PL1)

Regardless of the algal cell concentrations tested, *R. reticulata* either in combination with other algal species (except with *S. costatum*) or alone did not support larval survival beyond PZ3/M1 stage (Tables 3-4). Hence, statistical analyses were only conducted on larval survival and growth obtained by using the algal feeds which promoted survival until PL1 at cell densities between 30 and 50 cells μl^{-1}. *P. indicus* larvae were fed five newly hatched *Artemia* ml^{-1} together with test algal feeds between M1 to PL1 stages. Two-way ANOVA results on larval survival (Table 13a) indicate that there was no significant interaction between algal feeds and cell concentrations on larval survival from M1 to PL1. Hence, no further statistical analysis was carried out for the effect of cell densities and/ or algal feeds independently on larval survival at these stages. However, it was necessary to perform one-way analysis of variances separately for algal feeds and cell concentrations following two-way ANOVA (Table 14a) as there was significant interaction between algal feeds and cell concentrations on larval growth at PL1 stage ($P<0.001$).

Figures 2a and 2b show that highest mean larval survival (59 %) and larval growth (5.34 mm TL) at cell densities from 30-50 cells μl^{-1} was promoted by the mixed algae (*T. chuii* / *S. costatum*) plus five *Artemia* ml^{-1} ($P<0.05$) between M1 and PL1 stage (Tables 13b and 14b). There was no significant difference ($P>0.05$) between mean larval survival and larval growth supported by *T. chuii* and *S. costatum* as single diets (Tables 13b, 14b). Increase in algal cell densities from 30 to 40 and 50 cells μl^{-1} increased larval survival and larval growth from 20 %, 4.81 mm TL to 37 %, 5.04 mm TL, and 48 %, 5.32 mm TL respectively ($P<0.05$) (see Tables 13c and 14 c).

<u>30 cells μl^{-1}</u>

Any significant difference in larval survival and growth on this algal cell concentration is summarised in Figures 2a, b. Following two-way ANOVA,

further analysis of variance (one-way ANOVA) on larval growth at 30 cells μl^{-1} indicates that highest larval growth at PL1 (5.05 mm TL) was supported by the mixed algae (*T. chuii* / *S. costatum*) and *Artemia* (Tables 15a, b). There was no significant ($P<0.05$) difference between larval growth or survival promoted by different single algal diets plus *Artemia* (see Figures 2a, b). As shown in Figure 2a, the highest larval survival (45 %) at PL1 was obtained from the mixed algal diet plus five *Artemia* ml^{-1} ($P<0.05$).

40 cells μl^{-1}

Figure 2b shows that the mixed algae (*T. chuii* / *S. costatum*) gave better larval growth (5.33 mm TL) than *T. chuii* (4.82 mm TL) or *S. costatum* (4.96 mm TL) alone at 40 cells μl^{-1} at PL1 stage (Tables 16a, b). Mean growth attained by larvae fed *T. chuii* was significantly smaller ($P>0.05$) than those fed *S. costatum* until PL1 stage. Figure 2a demonstrates that larvae fed the mixed algae showed significantly higher survival (55 %) than those fed either of the single algal species (see also Table 4a). There was no significant difference between survival of the larvae fed either of the single algal species (Figure 2a).

50 cells μl^{-1}

Highest larval growth at 50 cells μl^{-1} (5.65 mm TL) was again attained on the mixed algae (*T. chuii* / *S. costatum*) (see Figure 2b and Tables 17a, b). Figure 2a shows that larvae fed the mixed algae had significantly higher survival (77 %) than those fed *S. costatum* (42 %) and *T. chuii* (27 %) alone. Scheffé's multiple pairwise comparison test (Table 17b) shows that the effect of single algal species on larval growth did not differ significantly from each other ($P>0.05$). Survival of larvae fed *S. costatum* between M1 and PL1 along with *Artemia* was also not significantly higher than that of *T. chuii*. Table 13c shows that larval survival at this cell concentration was significantly better than the larvae fed on either 30 or 40 cells μl^{-1} ($P<0.05$).

Figure 2a

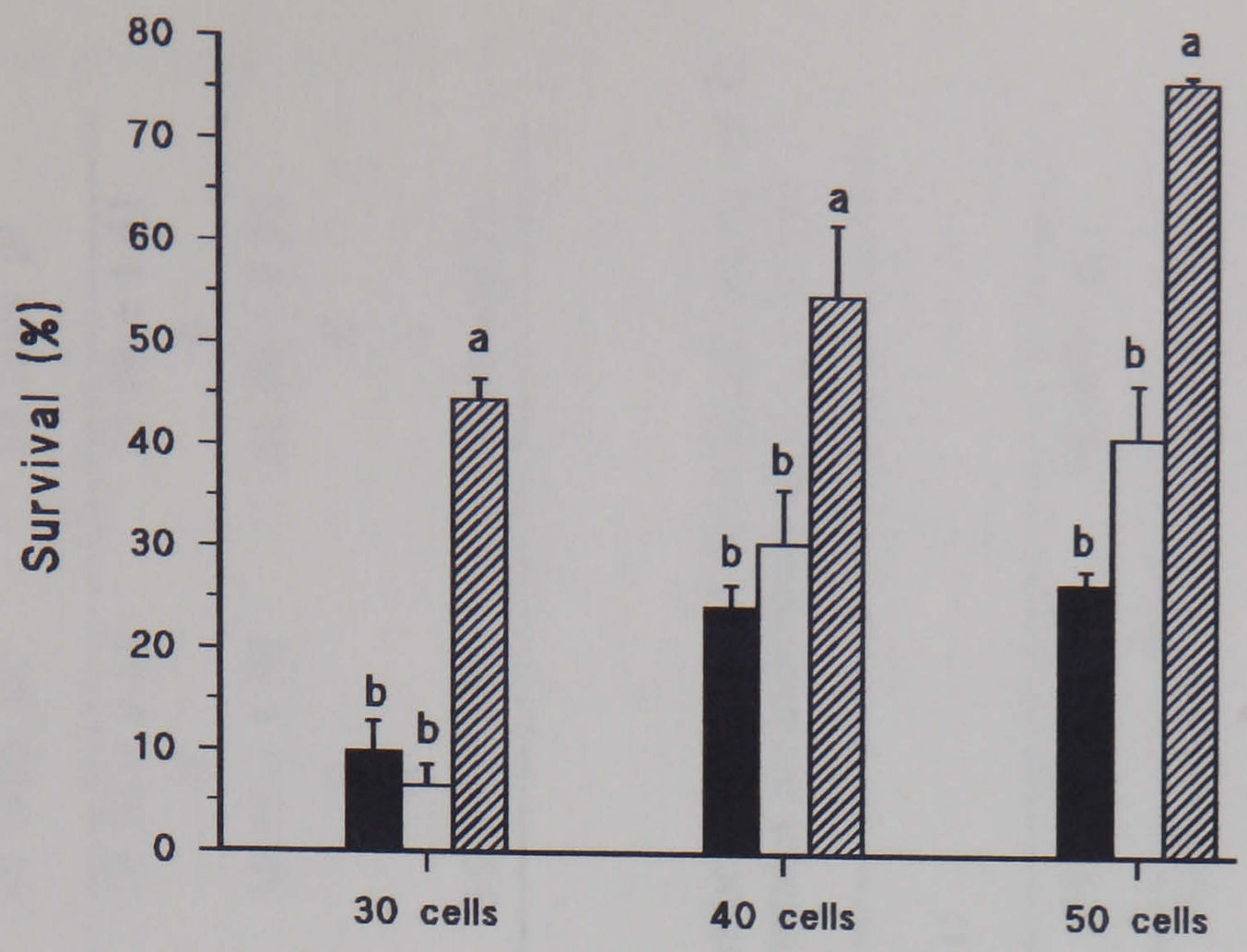


Figure 2b

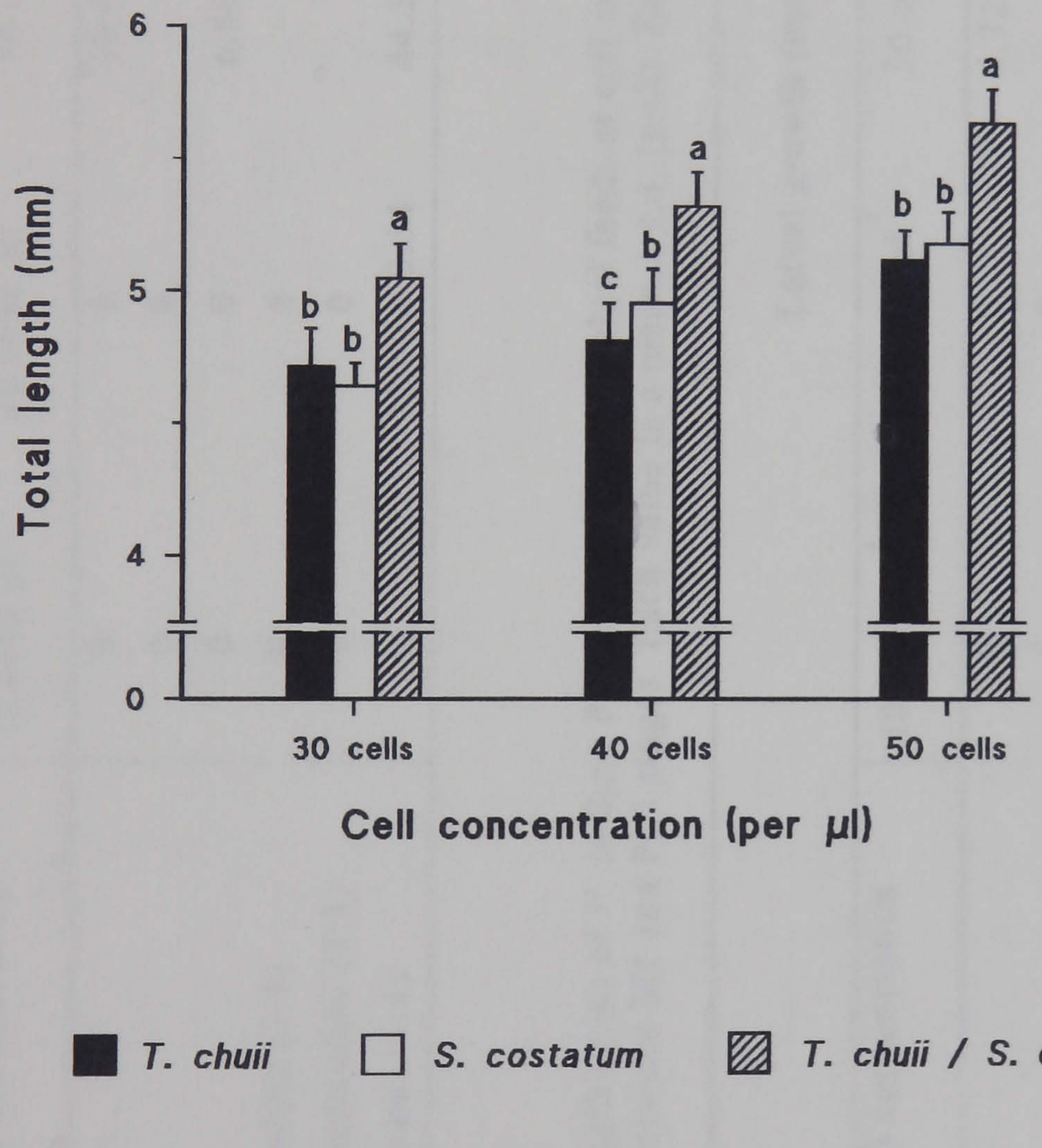


Figure 2. (a) Larval survival (%) and (b) growth (mm TL) of *P. indicus* reared on three algal combinations (*T. chuii*, *S. costatum* and *T. chuii / S. costatum*) at three algal cell concentrations of 30, 40 and 50 cells μl^{-1} plus five *Artemia* ml^{-1} between M1and PL stages. Each bar represents a mean ± s.d. (n=2) for at PL1 stage. Each replicate contains measurements of 10-13 larvae for growth. Treatments marked with different superscripts are significantly different ($P<0.05$).

Table 4a. Survival (%) of *P. indicus* PL1 larvae reared on algal feeds at cell concentrations of between 10 and 50 cells μl^{-1} along with five *Artemia* ml^{-1} between M1 and PL1 stages. Each value is a mean ± s.d. (n=2).

	Larval survival (%) ± s.d. (PL1)				
Algal species/Cell concentration	10 cells μl^{-1}	20 cells μl^{-1}	30 cells μl^{-1}	40 cells μl^{-1}	50 cells μl^{-1}
T. chuii	0	0	10.00 ± 2.83	24.50 ± 2.12	27.00 ± 1.41
R. reticulata	0	0	0	0	0
S. costatum	0	0	6.50 ± 2.12	30.75 ± 5.30	41.50 ± 4.95
T. chuii / R. reticulata (1:1)	0	0	0	0	0
S. costatum / R. reticulata (1:1)	0	0	0	0	0
T. chuii / S. costatum (1:1)	0	11.00 ± 3.54	44.50 ± 2.12	55.00 ± 7.07	76.50 ± 0.71

Table 4b. Total length (mm) of *P. indicus* PL1 larvae reared on algal feeds at cell concentrations of between 10 and 50 cells μl^{-1} along with five *Artemia* ml^{-1} between M1 and PL1 stages. Each value is a mean ± s.d. (n=2). Each replicate contains measurement of 10-13 larvae.

	Larval growth (mm) ± s.d. (PL1)				
Algal species/Cell concentration	10 cells μl^{-1}	20 cells μl^{-1}	30 cells μl^{-1}	40 cells μl^{-1}	50 cells μl^{-1}
T. chuii	0	0	4.72 ± 0.14	4.82 ± 0.14	5.13 ± 0.11
R. reticulata	0	0	0	0	0
S. costatum	0	0	4.64 ± 0.09	4.96 ± 0.13	5.19 ± 0.12
T. chuii / R. reticulata (1:1)	0	0	0	0	0
S. costatum / R. reticulata (1:1)	0	0	0	0	0
T. chuii / S. costatum (1:1)	0	4.95 ± 0.06	5.05 ± 0.13	5.33 ± 0.13	5.65 ± 0.13

a) Survival and growth (PZ1-PZ3/M1)

(***) Denotes pairs of groups significantly different ($P \leq 0.001$).
(**) Denotes pairs of groups significantly different ($P \leq 0.01$).
(*) Denotes pairs of groups significantly different ($P \leq 0.05$).
(Ns) Indicates a non-significant difference ($P>0.05$).

Table 5a. Two-way ANOVA on survival from PZ1 to PZ3/M1 stages by algal feeds and cell concentrations.

Source	DF	SS	MS	F	P	Signifi-cant
Algae	2	38.72.86	1936.43	199.18	0.000	***
Cells	2	2708.11	1354.06	139.27	0.000	***
Algae*Cells	4	254.97	63.74	6.56	0.009	**
Error	9	87.50	9.72			
Total	17	6923.44				

Table 5b. Tukey's pairwise comparison test on larval survival from PZ1 to PZ3/M1 on three different live algal diets.

Algal feeds	Code	Means (%)	Test	diffmean	low_ci	up_ci	Signifi-cant
T. chuii	Tc	29.75	Tc-Sc	-4.833	-9.861	0.195	Ns
S. costatum	Sc	34.58	Tc/Sc-Tc	-33.250	-38.278	-28.223	*
T. chuii /S. costatum	Tc/Sc	63.00	Tc/Sc-Sc	-28.417	-33.445	-23.390	*

Table 5c. Tukey's pairwise comparison test on larval survival from PZ1 to PZ3/M1 on three cell concentrations (30-50 cells μl^{-1}).

Code	Cells μl^{-1}	Means (%)	Test	diffmean	low_ci	up_ci	Signifi-cant
A	30 cells	26.50	A-B	-18.000	-23.028	-12.973	*
B	40 cells	44.50	A-C	-29.833	-34.861	-24.806	*
C	50 cells	56.33	B-C	-11.833	-16.861	-6.806	*

Table 6a. One-way ANOVA on larval survival (PZ1-PZ3/M1) by algal feeds at cell concentration of 30 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Cells	2	1729.00	864.50	178.86	0.001	***
Error	3	14.50	4.83			
Total	5	1743.50				

Bartlett's test for homogeneity of variances
$F = 0.312$, $P = 0.14$

Table 6b. Tukey's pairwise comparison test on larval survival (PZ1-PZ3/ M1) by algal feeds at cell concentration of 30 cells μl^{-1}.

Mean (%)	Algal feeds	Code	Tc	Sc	Tc/Sc
15.00	*T. chuii*	Tc			
14.00	*S. costatum*	Sc	NS		
50.50	*T. chuii / S. costatum*	Tc/Sc	*	*	

Table 7a. One-way ANOVA on larval survival (PZ1-PZ3/M1) by algal feeds at cell concentration of 40 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Cells	2	508.0	254.0	18.36	0.021	*
Error	3	41.5	13.8			
Total	5	549.5				

Bartlett's test for homogeneity of variances
F = 0.452, P = 0.45

Table 7b. Tukey's pairwise comparison test on larval survival (PZ1-PZ3/M1) by algal feeds at cell concentration of 40 cells μl^{-1}.

Mean (%)	Algae	Code	Tc	Sc	Tc/Sc
38.50	*T. chuii*	Tc			
39.50	*S. costatum*	Sc	NS		
58.50	*T. chuii / S. costatum*	Tc/Sc	*	*	

Table 8a. One-way ANOVA on larval survival (PZ1-PZ3/M1) by algal feeds at cell concentration of 50 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Cells	2	1771.6	885.8	57.46	0.004	**
Error	3	46.2	15.4			
Total	5	1817.8				

Bartlett's test for homogeneity of variances
F = 0.329, P = 0.15

Table 8b. Tukey's pairwise comparison test on larval survival (PZ1-PZ3 /M1) by algal feeds at cell concentration of 50 cells μl^{-1}.

Mean (%)	Algae	Code	Tc	Sc	Tc/Sc
39.00	*T. chuii*	Tc			
50.25	*S. costatum*	Sc	NS		
79.75	*T. chuii / S. costatum*	Tc/Sc	*	*	

Table 9a. Two-way ANOVA on growth of *P. indicus* larvae reared on *T. chuii* (Tc) / *S. costatum* (Sc), Tc and Sc singly (30, 40 and 50 cells μl^{-1}) from PZ1 to PZ3/M1 stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Algae	2	30.9455	30.2768	15.1384	618.56	0.000	***
Cells	2	24.7998	24.7501	12.3751	505.65	0.000	***
Algae*Cells	4	5.5059	5.5059	1.3765	56.24	0.000	***
Error	170	4.1605	4.1605	0.0245			
Total	178	65.4117					

Table 9b. Scheffé's pairwise comparison test on larval growth by algal species (PZ1-PZ3/M1).

Algal feeds	Code	Means (mm)	Test	diffmean	SE	low_ci	up_ci	Signifi-cant
T. chuii	Tc	2.706	Tc-Sc	-0.555	0.0287	-0.626	-0.484	*
S. costatum	Sc	3.261	Tc-Tc/Sc	-1.013	0.0288	-1.084	-0.942	*
T. chuii /S. *costatum*	Tc/Sc	3.719	Tc/Sc-Sc	-0.458	0.0284	-0.528	-0.388	*

Table 9c. Scheffé's pairwise comparison test on larval growth (PZ1-PZ3/M1) by cell concentrations (30-50 cells μl^{-1}).

Code	Cells (μl^{-1})	Means (mm)	Test	diffmean	SE	low_ci	up_ci	Signifi-cant
A	30 cells	2.771	A-B	-0.463	0.029	-0.534	-0.392	*
B	40 cells	3.234	A-C	-0.909	0.029	-0.979	-0.838	*
C	50 cells	3.680	B-C	-0.446	0.029	-0.517	-0.375	*

Table 10a. One-way ANOVA on larval growth (PZ1-PZ3/M1) by algal feeds at cell density of 30 cells μl^{-1}.

SOURCE	DF	SS	MS	F	P	Signifi-cant
Algae	2	4.0785	2.0393	156.91	0.000	***
Error	57	0.7408	0.0130			
Total	59	4.8193				

Bartlett's test for homogeneity of variance

F = 4.832, P= 0.92

Table 10b. Scheffé's pairwise comparison test on larval growth (PZ1-PZ3/M1) by algal feeds at 30 cells μl^{-1}.

Mean (mm)	Algal feeds	Code	Tc	Sc	Tc/Sc
2.542	*T. chuii*	Tc			
2.591	*S. costatum*	Sc	NS		
3.138	*T. chuii* / *S. costatum*	Tc/Sc	*	*	

Table 11a. One-way ANOVA on larval growth (PZ1-PZ3/M1) by algal feeds at cell concentration of 40 cells μl^{-1}.

SOURCE	DF	SS	MS	F	P	Signifi-cant
Algae	2	9.7071	4.8536	263.15	0.000	***
Error	57	1.0513	0.0184			
Total	59	10.7584				

Bartlett's test for homogeneity of variance
F = 3.532, P = 0.83

Table 11b. Scheffé's pairwise comparison test on larval growth (PZ1-PZ3/M1) by algal feeds at cell density of 40 cells μl^{-1}.

Mean (mm)	Algal feeds	Code	Tc	Sc	Tc/Sc
2.712	*T. chuii*	Tc			
3.334	*S. costatum*	Sc	*		
3.694	*T. chuii* / *S. costatum*	Tc/Sc	*	*	

Table 12a. One-way ANOVA on larval growth (PZ1-PZ3/M1) by algal feeds at cell concentration of 50 cells μl^{-1}.

SOURCE	DF	SS	MS	F	P	Significant
Algae	2	2.4251	1.2126	82.42	0.000	***
Error	57	0.8386	0.0147			
Total	59	3.2638				

Bartlett's test for homogeneity of variances
F = 2.499, P = 0.71

Table 12b. Scheffé's pairwise comparison test on larval growth (PZ1-PZ3/M1) by algal feeds at cell concentration of 50 cells μl^{-1}.

Mean (mm)	Algal feeds	Code	Tc	Sc	Tc/Sc
3.591	*T. chuii*	Tc			
3.924	*S. costatum*	Sc	*		
4.081	*T. chuii* / *S. costatum*	Tc/Sc	*	*	

b) Survival and growth (M1-PL1)

(***) Denotes pairs of groups significantly different ($P \leq 0.001$).
(**) Denotes pairs of groups significantly different ($P \leq 0.01$).
(*) Denotes pairs of groups significantly different ($P \leq 0.05$).
(Ns) Indicates a non-significant difference ($P>0.05$).

Table 13a. Two-way ANOVA on larval survival at PL1 stage by algal species and cell concentrations plus five *Artemia* ml^{-1}.

Source	DF	SS	MS	F	P	Signifi-cant
Algae	2	5081.19	2540.60	111.74	0.000	***
Cells	2	2.375.36	1187.68	52.24	0.000	***
Algae*Cells	4	311.72	77.93	3.43	0.058	Ns
Error	9	204.63	22.74			
Total	17	7972.90				

Table 13b. Tukey's pairwise comparison test on larval survival (PL1) by three live algal feeds with five *Artemia* ml^{-1}.

Algal feeds	Code	Means (%)	Test	diffmean	low_ci	up_ci	Signifi-cant
T. chuii	Tc	20.50	Tc-Sc	-5.75	-13.440	1.940	Ns
S. costatum	Sc	26.25	Tc-Tc/Sc	-38.167	-45.856	-30.478	*
T. chuii / *S. costatum*	Tc/Sc	58.67	Sc-Tc/Sc	-32.417	-40.107	-24.727	*

Table 13c. Tukey's pairwise comparison test on larval survival (PL1 by algal cell concentrations with five *Artemia* ml^{-1}.

Code	Cells (μl^{-1})	Means (%)	Test	diffmean	low_ci	up_ci	Signifi-cant
A	30 cells	20.33	A-B	-16.417	-24.407	-8.727	*
B	40 cells	36.78	A-C	-28.000	-35.690	-20.310	*
C	50 cells	48.33	B-C	-11.583	-19.273	-3.893	*

Table 14a. Two-way ANOVA on growth (mm) of *P. indicus* larvae (PL1) by algal feeds and cell concentrations plus five *Artemia* ml^{-1} .

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Diets	2	7.4221	7.8000	3.9000	251.27	0.000	***
Cells	2	8.5213	8.5559	4.2779	275.63	0.000	***
Diets*Cells	4	0.3654	0.3654	0.0913	5.89	0.000	***
Error	181	2.8093	2.8093	0.0155			
Total	189	19.1181					

Table 14b. Scheffé's pairwise comparison test on larval growth (PL1) by algal feeds plus five *Artemia* nauplii ml^{-1} .

Algal feeds	Code	Means (mm)	Test	diffmean	SE	low_ci	up_ci	Signifi-cant
T. chuii	Tc	4.893	Tc-Sc	-0.035	0.0219	-0.089	0.019	NS
S. costatum	Sc	4.928	Tc/Sc-Tc	-0.451	0.0223	-0.506	-0.396	*
T. chuii /S. costatum	Tc/Sc	5.344	Tc/Sc-Sc	-0.416	0.0222	-0.471	-0.361	*

Table 14c. Scheffé's test on larval growth (PL1) by three cell concentrations plus five *Artemia* nauplii ml^{-1} .

Code	Cells (μl^{-1})	Means (mm)	Test	diffmean	SE	low_ci	up_ci	Signifi-cant
A	30 cells	4.805	A-B	-0.231	0.0219	-0.285	-0.177	*
B	40 cells	5.036	A-C	-0.519	0.0221	-0.574	-0.464	*
C	50 cells	5.324	B-C	-0.288	0.0224	-0.343	-0.233	*

Table 15a. One-way ANOVA on larval growth at PL1 by algal feeds at cell concentration of 30 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	2	2.1331	1.0665	69.09	0.000	***
Error	63	0.9726	0.0154			
Total	65	3.1056				

Bartlett's test for homogeneity of variances
F = 3.836, P = 0.93

Table 15b. Scheffé's pairwise comparison test on larval growth (PL1) by algal feeds at cell concentration of 30 cells μl^{-1}.

Mean (mm)	Algae	Code	Tc	Sc	Tc/Sc
4.723	*T. chuii*	Tc			
4.636	*S. costatum*	Sc	NS		
5.054	*T. chuii / S. costatum*	Tc/Sc	*	*	

Table 16a. One-way ANOVA on larval growth at PL1 by algal feeds at cell concentration of 40 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	2	2.8177	1.4088	84.08	0.000	***
Error	60	1.0054	0.0168			
Total	62	3.8231				

Bartlett's test for homogeneity of variances
F = 0.099, P = 0.52

Table 16b. Scheffé's pairwise comparison test on larval growth (at PL1) by algal feeds at cell concentration of 40 cells μl^{-1}.

Mean (mm)	Algae	Code	Tc	Sc	Tc/Sc
4.823	*T. chuii*	Tc			
4.956	*S. costatum*	Sc	*		
5.330	*T. chuii* / *S. costatum*	Tc/Sc	*	*	

Table 17a. One-way ANOVA on larval growth at PL1 by algal feeds at cell concentration of 50 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	2	3.1373	1.5687	109.45	0.000	***
Error	58	0.8313	0.0143			
Total	60	3.9686				

Bartlett's test for homogeneity of variances
F = 0.364, P = 0.58

Table 17b. Scheffé's pairwise comparison test on larval growth (at PL1) by algal feeds at cell concentration of 50 cells μl^{-1}.

Mean (mm)	Algae	Code	Tc	Sc	Tc/Sc
5.132	*T. chuii*	Tc			
5.192	*S. costatum*	Sc	Ns		
5.649	*T. chuii* / *S. costatum*	Tc/Sc	*	*	

Experiment 2

(a) Growth and survival (PZ1-PZ3/M1)

From Experiment 1 it appears that the algal feeds giving best larval growth and survival of *P. indicus* were those supplied at the highest cell densities. Thus, in the present experiment higher cell concentrations (50, 60, 70 and 80 cells μl^{-1}) of the diatom *S. costatum* and the flagellate *T. chuii* singly and in combination were fed to the larvae from PZ1 to PL stages. Growth and survival results were analysed by two-way ANOVA followed by one-way ANOVA independently for algal feeds and cell concentrations when there were significant interactions between algal feeds and cell concentrations on larval growth and survival. Significant differences were determined using Scheffé's and Tukey's multiple pairwise comparison tests.

Figures 3a, b plot survival and growth of *P. indicus* larvae fed on two algal feeding regimes at cell densities of between 50 and 80 cells μl^{-1} at PZ3/M1 stages. Tables 18a and 23a show that there were significant differences between the effects of feeds on larval growth and survival at different cell concentrations at PZ1-PZ3/M1 ($P<0.001$). Greater total length ($P<0.001$) and higher survival ($P<0.001$) were obtained on mixed algae (*T. chuii* / *S. costatum*) than with the diatom (*S. costatum*) singly. Higher cell densities (70-80 cells μl^{-1}) sustained better larval survival and growth compared to lower cell densities (50-60 cells μl^{-1}) between PZ1 and PZ3/M1 stages (Tables 18b, 23b). However there were significant interactions between larval feeds and cell densities on both survival and growth *P. indicus* larvae (see Tables 18a, 23a). Therefore, growth and survival data was further analysed by one-way ANOVA specifically for each cell concentrations. Growth and survival data at PZ3/M1 and PL stages are summarised in Tables 38 and 39.

50 cells μl^{-1}

Figures 3a, b and one-way ANOVA tables (Tables 19 and 24) show that the mixed algae (*T. chuii / S. costatum*) gave higher larval survival (67 %) and growth (3.54 mm TL) at 50 cells μl^{-1} than those obtained from larvae fed on *S. costatum* singly (50 % and 3.37 mm TL) from PZ1 to PZ3/M1 stages ($P<0.01$).

60 cells μl^{-1}

Tables 20 and 25 show that larvae fed mixed algae (*T. chuii / S. costatum*) supported almost twice as high survival (87.50 %) and significantly better growth (3.84 mm TL) ($P<0.001$) than the single algal diet (*S. costatum*) which gave only 45.5 % survival and 3.51 mm TL at PZ3/M1 stages (see also Figures 3 a, b).

70 cells μl^{-1}

Figures 3a, b demonstrate that larval growth (3.98 mm TL) promoted by the mixed algae (*T. chuii / S. costatum*) was significantly better than that (3.79 mm TL) of *S. costatum* alone at 70 cells μl^{-1} ($P<0.001$) (Table 26). However, survivals of larvae fed on either of the algal feeds were not significantly ($P>0.05$) different at PZ3/M1 stages (Table 21).

80 cells μl^{-1}

Larvae fed on the mixed algae (*T. chuii / S. costatum*) showed better growth ($P<0.05$) than *S. costatum* alone at this cell concentration (Table 27). Larval survivals, however, did not differ on either of the algal diets at PZ3/M1 stages at 0.05 significance level (Table 22). *S. costatum* at cell densities of both 70 and 80 cells μl^{-1} tended to aggregate in the larval culture water causing larval fouling. A number of larvae were observed to stick together with their faecal strings.

Figure 3a

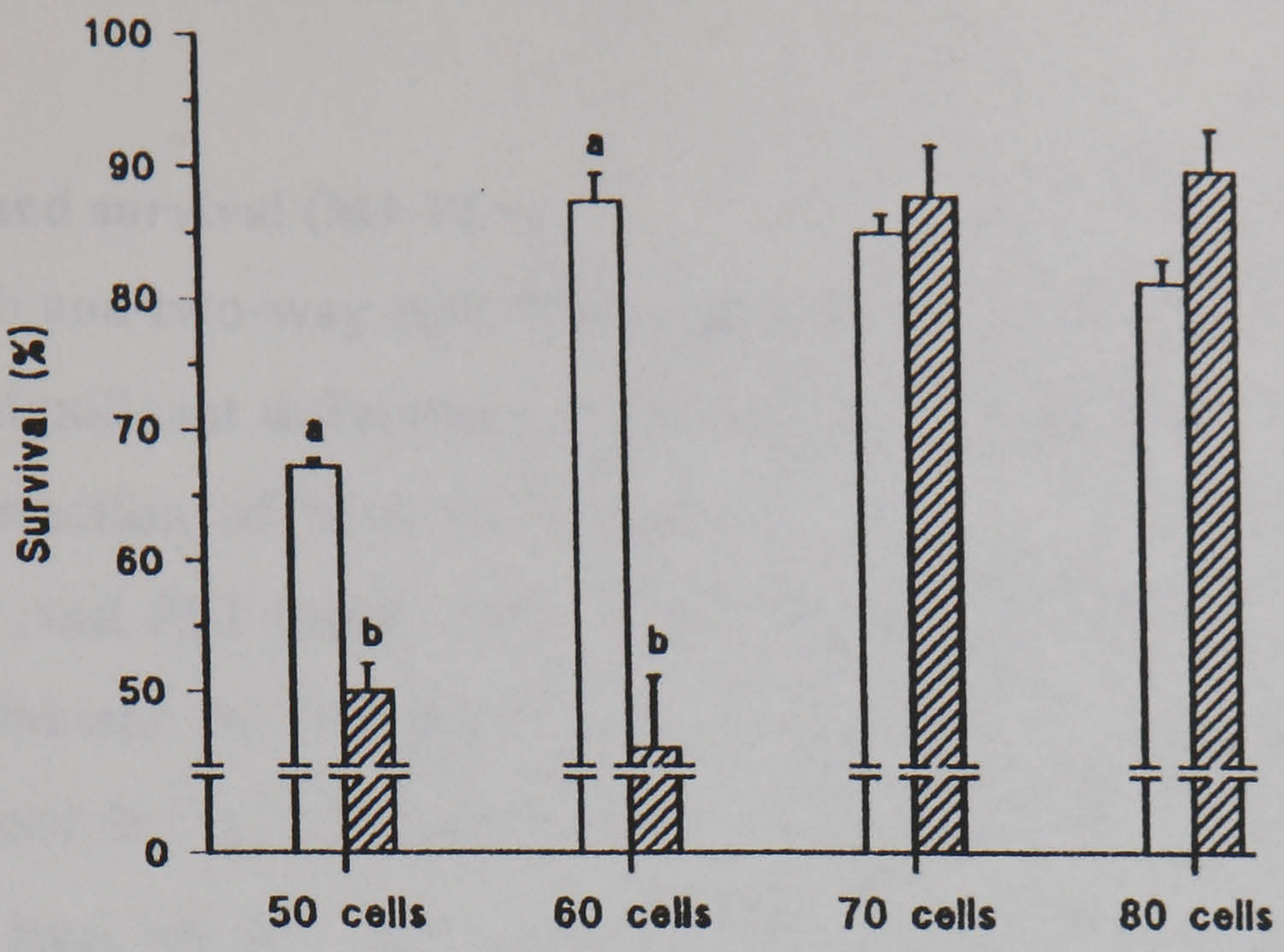


Figure 3b

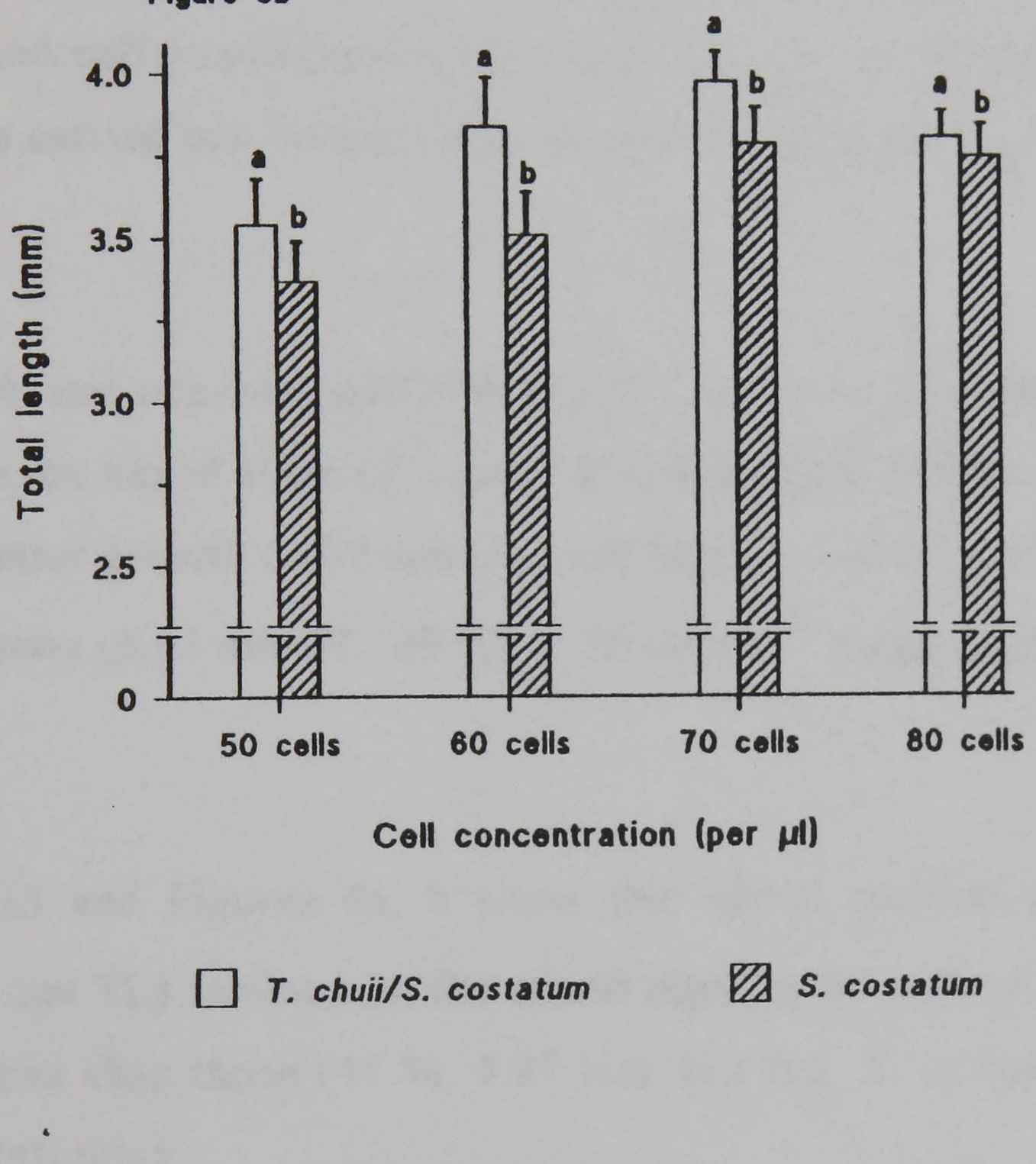


Figure 3. (a) Larval survival (%) and (b) growth (mm TL) of *P. indicus* reared on two feeding regimes (*T. chuii* and *T. chuii* / *S. costatum*) at four algal cell concentrations of 50, 60, 70 and 80 cells μl^{-1} from PZ1 to PZ3/M1 stages. Each bar represents a mean ± s.d. (n=2). Each replicate contains measurements of 10-13 larvae for growth. Treatments marked with different superscripts are significantly different ($P<0.05$).

(b) Growth and survival (M1-PL1)

Figures 4a, b and two-way ANOVA results show that (Tables 28a and 33a) there were significant differences in the effects of algal feeds, cell densities and the interaction of both on larval survival and growth of *P. indicus* between M1 and PL1 stages ($P<0.001$). Mixed algae (*T. chuii* / *S. costatum*) plus *Artemia* fed larvae attained higher survival and greater size at PL1 than those fed on *S. costatum* plus *Artemia* alone (Figures 4a, b). Regardless of algal species, 80 cells μl^{-1} cell concentrations supported better growth but significantly inferior survival than lower cell densities (see Figures 4a, b and Tables 28b, 33b). Highest survival was promoted by 70 cells μl^{-1} at PL1 stage. Since there were interactions between the effect of algal feeds and cell concentrations on larval growth and survival, one-way ANOVA was carried out for each cell concentration of the algal diets.

<u>50 cells μl^{-1}</u>

Figures 4a, b and one-way ANOVA tables (Tables 29 and 34) show that larvae fed on the mixed algae (*T. chuii* / *S. costatum*) exhibited significantly ($P<0.001$) better growth (6.07 mm TL) and higher survival (52%) ($P<0.05$) than *S. costatum* (5.65 mm TL, 39 %) at 50 cells μl^{-1} concentration.

<u>60 cells μl^{-1}</u>

Tables 30, 35 and Figures 4a, b show that larval survival (69 %) and growth (6.1 mm TL) attained on the mixed algae at 60 cells μl^{-1} were significantly better than those (41 %, 5.87 mm TL) fed *S. costatum* alone at PL1 stage ($P<0.001$).

<u>70 cells μl^{-1}</u>

Table 31 shows that survival of larvae fed on the mixed algae and single algal diet was not significantly different at this cell concentration ($P>0.05$). However, Table 36 and Figure 4b show that larval growth attained on the

mixed algae (*T. chuii* / *S. costatum*) was significantly better (6.33 mm TL) than that (5.82 mm TL) on the diatom *S. costatum* ($P<0.001$).

80 cells μl^{-1}

Larval survival (58.5%) and growth (6.28 mm TL) on the mixed algae at this cell density were significantly better than those (28 %, 6.16 mm TL) obtained from the single algal diet ($P<0.05$) (see Tables 32, 37 and Figures 4a, b). As in protozoeal stages, when *S. costatum* was fed to the larvae at this cell density and at 70 cells μl^{-1} fouling on the appendages of the larvae caused mortality.

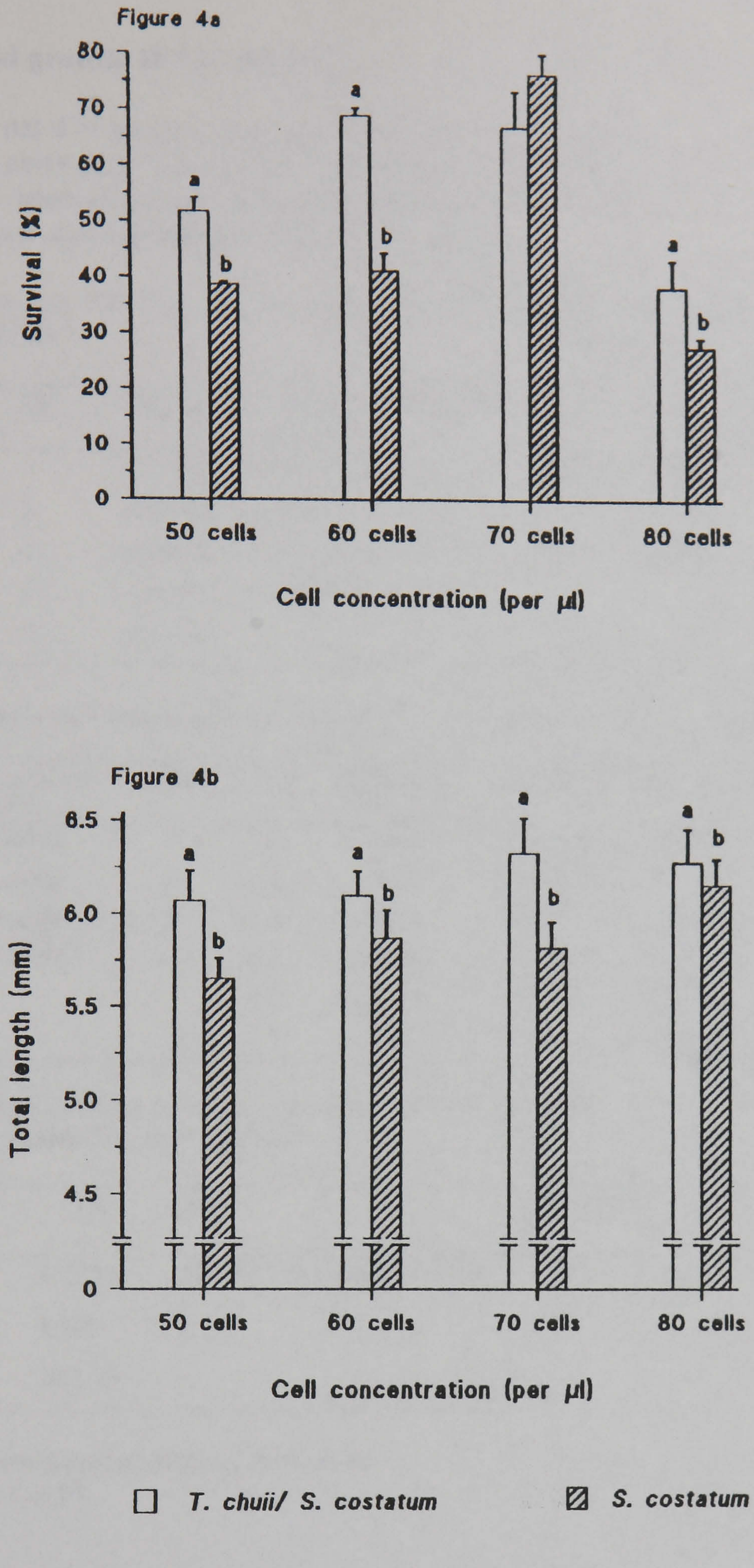


Figure 4. (a) Larval survival (%) and (b) growth (mm TL)) of *P. indicus* reared on two feeding regimes (*T. chuii* and *T. chuii / S. costatum*) at four algal cell concentrations of 50, 60, 70 and 80 cells μl^{-1} and five *Artemia* ml^{-1} between M1 and PL1 stages. Each bar represents a mean ± s.d. (n=2). Each replicate contains measurements of 10-13 larvae for growth. Treatments marked with different superscripts are significantly different ($P<0.05$).

a) Survival and growth (PZ1-PZ3/M1)

(***) Denotes pairs of groups significantly different ($P \leq 0.001$).
(**) Denotes pairs of groups significantly different ($P \leq 0.01$).
(*) Denotes pairs of groups significantly different ($P \leq 0.05$).
(Ns) Indicates a non-significant difference ($P>0.05$).

Table 18a. Two-way ANOVA on larval survival at PZ3/M1 by algal feeds at different cell concentrations.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Algae	1	564.06	564.06	564.06	60.57	0.000	***
Cells	3	2288.81	2288.81	762.94	81.93	0.000	***
Algae*Cells	3	1586.06	1586.06	528.69	56.77	0.000	***
Error	8	74.50	74.50	9.31			
Total	15	4513.44					

Table 18b. Tukey's pairwise comparison test on larval survival by algal cell concentrations.

Means (%)	Cell concent. (μl^{-1})	Code	Test	diffmean	low_ci	up_ci	Signifi-cant
85.25	80 cells	1	1-2	- 6.13	-13.041	0.781	Ns
91.38	70 cells	2	1-2	18.75	11.839	25.661	*
66.50	60 cells	3	1-4	26.62	19.709	33.531	*
58.63	50 cells	4	2-3	24.88	17.969	31.791	*
			2-4	32.75	25.839	39.661	*
			3-4	7.87	0.959	14.781	*

Table 19. One-way ANOVA on larval survival at PZ3/M1 by algal feeds at cell concentration of 50 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	297.56	297.56	128.68	0.008	**
Error	2	4.62	2.31			
Total	3	302.19				

Bartlett's test for homogeneity of variances
$F = 1.005$, $P = 0.24$

Table 20. One-way ANOVA on larval survival at PZ3/M1 by algal feeds at cell concentration of 60 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	1764.0	1764.0	96.66	0.010	**
Error	2	36.5	18.3			
Total	3	1800.5				

Bartlett's test for homogeneity of variances
$F = 0.560$, $P = 0.40$

Table 21. One-way ANOVA on larval survival at PZ3/M1 by algal feeds at cell concentration of 70 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	7.56	7.56	0.88	0.447	Ns
Error	2	17.13	8.56			
Total	3	24.69				

Bartlett's test for homogeneity of variances
F = 0.594, P = 0.38

Table 22. One-way ANOVA on larval survival at PZ3/M1 by algal feeds at cell concentration of 80 cells μl^{-1}.

DF	SS	MS	F	P	Significant
1	81.00	81.00	9.97	0.087	Ns
2	16.25	8.12			
3	97.25				

Bartlett's test for homogeneity of variances
F = 0.218, F = 0.77

Table 23a. Two-way ANOVA on larval growth at PZ3/M1 by algal feeds at different cell concentrations.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Algae	1	1.46998	1.43641	1.43641	83.59	0.000	***
Cells	3	4.70593	4.71358	1.57119	91.44	0.000	***
Algae*Cells	3	0.34465	0.34465	0.11488	6.69	0.000	***
Error	171	2.93830	2.93830	0.01718			
Total	178	9.45886					

Table 23b. Scheffé's pairwise comparison test on larval growth at different algal cell concentrations.

Cell Conc. (μl^{-1})	Means (mm)	Code	Test	diffmean	SE	low_ci	up_ci	Signifi-cant
80 cells	3.78	1	1-2	-0.108	0.0282	-0.1878	-0.0281	*
70 cells	3.60	2	1-3	0.117	0.0279	0.0388	0.1951	*
60 cells	3.66	3	1-4	0.331	0.0281	0.2515	0.4104	*
50 cells	3.45	4	2-3	0.225	0.0273	0.1477	0.3022	*
			2-4	0.439	0.0277	0.3605	0.5174	*
			3-4	0.214	0.0271	0.1372	0.2907	*

Table 24. One-way ANOVA on larval growth at PZ3/M1 by algal feeds at cell concentration of 50 cells μl^{-1}.

Source	DF	SS	MS	F	P	Signifi-cant
Cells	1	0.3153	0.3153	18.95	0.000	***
Error	43	0.7154	0.0166			
Total	44	1.0307				

Bartlett's test for homogeneity of variances
F = 0.729, P = 0.32

Table 25. One-way ANOVA on larval growth at PZ3/M1 by algal feeds at cell concentration of 60 cells μl^{-1}.

Source	DF	SS	MS	F	P	Signifi-cant
Algae	1	1.2156	1.2156	40.28	0.000	***
Error	42	1.2675	0.0302			
Total	43	2.4831				

Bartlett's test for homogeneity of variances
F = 0.420, P = 0.50

Table 26. One-way ANOVA on larval growth at PZ3/M1 by algal feeds at cell concentration of 70 cells μl^{-1}.

Source	DF	SS	MS	F	P	Signifi-cant
Algae	1	0.3856	0.3856	34.56	0.000	***
Error	42	0.4668	0.0111			
Total	43	0.8524				

Bartlett's test for homogeneity of variances
F = 1.593, P = 0.15

Table 27. One-way ANOVA on larval growth at PZ3/M1 by algal feeds at cell concentration of 80 cells μl^{-1}.

Source	DF	SS	MS	F	P	Signifi-cant
Algae	1	0.03544	0.03544	4.11	0.049	*
Error	42	0.34472	0.00862			
Total	41	0.38016				

Bartlett's test for homogeneity of variances
F = 0.964, P = 0.75

b) Survival and growth (M1-PL1)

Table 28a. Two-way ANOVA on larval survival (at PL1) by algal feeds at various cell densities (50-80 cells μl^{-1}) plus five *Artemia* ml^{-1} after PZ3/M1 stage.

Source	DF	SS	MS	F	P	Significant
Algae	1	992.25	992.25	88.20	0.000	***
Cells	3	2039.56	679.85	60.43	0.000	***
Algae*Cells	3	1039.13	346.37	30.79	0.000	***
Error	8	90.00	11.25			
Total	15	4160.94				

Table 28b. Tukey's pairwise comparison test on larval survival (PL1) by algal cell concentrations.

Code	Cell concent. (μl^{-1})	Means (%)	Test	diffmean	low_ci	up_ci	Signifi-cant
1	80 cells	43.13	1-2	-28.625	-36.222	-21.028	*
2	70 cells	71.75	1-3	-11.500	-19.097	-3.903	*
3	60 cells	54.63	1-4	-2.125	-9.722	5.472	Ns
4	50 cells	42.25	2-3	17.125	9.528	24.722	*
			2-4	26.500	18.903	34.097	*
			3-4	9.375	1.778	16.972	*

Table 29. One-way ANOVA on larval survival at PL1 by algal feeds at cell concentration of 50 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	169.00	169.00	54.08	0.018	*
Error	2	6.25	3.12			
Total	3	175.25				

Bartlett's test for homogeneity of variances
P = 1.696, P = 0.13

Table 30. One-way ANOVA on larval survival at PL1 by algal feeds at cell concentration of 60 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	826.56	826.56	136.34	0.007	**
Error	2	12.12	6.06			
Total	3	838.69				

Bartlett's test for homogeneity of variances
F = 0.398, P = 0.48

Table 31. One-way ANOVA on larval survival at PL1 by algal feeds at cell concentration of 70 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	90.2	90.2	3.41	0.206	Ns
Error	2	53.0	26.5			
Total	3	143.2				

Bartlett's test for homogeneity of variances
F = 0.218, P = 0.77

Table 32. One-way ANOVA on larval survival at PL1 by algal feeds at cell concentration of 80 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	945.6	945.6	68.46	0.014	*
Error	2	27.6	13.8			
Total	3	973.2				

Bartlett's test for homogeneity of variances
F = 0.609, P = 0.38

Table 33a. Two-way ANOVA on total length of *P. indicus* (at PL1 stage) reared on algal feed regimes plus *Artemia* from PZ3/M1 to PL1.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Algae	1	5.2048	4.7219	4.7219	210.02	0.000	***
Cells	3	3.2260	3.1980	1.0660	47.41	0.000	***
Algae*Cells	3	1.0559	1.0559	0.3520	15.65	0.000	***
Error	178	4.0020	4.002	0.0225			
Total	185	13.4889					

Table 33b. Scheffé's pairwise comparison test on larval growth by cell concentrations.

Means (mm)	Cell concent. (μl^{-1})	Code	Test	diffmean	SE	low_ci	up_ci	Signifi-cant
6.219	80 cells	1	1-2	0.145	0.0316	0.0557	0.2343	*
6.074	70 cells	2	1-3	0.233	0.0321	0.1422	0.3238	*
5.986	60 cells	3	1-4	0.358	0.0309	0.2707	0.4453	*
5.861	50 cells	4	2-3	0.088	0.0314	-0.0008	0.1768	Ns
			2-4	0.213	0.0301	0.1278	0.2982	*
			3-4	0.125	0.0307	0.0383	0.2117	*

Table 34. One-way ANOVA on larval growth at PL1 by algal feeds at cell concentration of 50 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	2.3242	2.3242	122.53	0.000	***
Error	50	0.9484	0.0190			
Total	51	3.2726				

Bartlett's test for homogeneity of variances
F = 2.784, P = 0.06

Table 35. One-way ANOVA on larval growth at PL1 by algal feeds at cell concentration of 60 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	0.5871	0.5871	33.89	0.000	***
Error	42	0.7276	0.0173			
Total	43	1.3147				

Bartlett's test for homogeneity of variances
F = 0.501, P = 0.44

Table 36. One-way ANOVA on larval growth at PL1 by algal feeds at cell concentration of 70 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	2.9901	2.9901	108.24	0.000	***
Error	45	1.2432	0.0276			
Total	46	4.2333				

Bartlett's test for homogeneity of variances
F = 2.828, P = 0.06

Table 37. One-way ANOVA on larval growth at PL1 by algal feeds at cell concentration of 80 cells μl^{-1}.

Source	DF	SS	MS	F	P	Significant
Algae	1	0.1535	0.1535	5.81	0.020	*
Error	41	1.0827	0.0264			
Total	42	1.2362				

Bartlett's test for homogeneity of variances
F = 0.912, P = 0.26

Table 38a. Larval survival (%) at PZ3/M1 stages of *P. indicus* fed two algal feeds. Each value represents a mean ± s.d. (n=2). (Data for Figure 3a).

Larval survival (%) ± s.d. (PZ3/M1)				
Algae/Cell concent.	50 cells μl^{-1}	60 cells μl^{-1}	70 cells μl^{-1}	80 cells μl^{-1}
T. chuii /S. costatum	67.26±0.53	87.50±2.12	85.00±1.41	81.25±1.77
S. costatum	50.00±2.12	45.50±5.66	87.75±3.89	89.75±3.18

Table 38b. Larval growth (mm TL) at PZ3/M1 stages of *P. indicus* fed two algal feeds. Each value represents a mean ± s.d. (n=2). (Data for Figure 3b).

Larval growth (mm) ± s.d. (PZ3/M1)				
Algae/Cell concent.	50 cells μl^{-1}	60 cells μl^{-1}	70 cells μl^{-1}	80 cells μl^{-1}
T. chuii /S. costatum	3.54±0.14	3.84±0.15	3.98±0.09	3.81±0.08
S. costatum	3.37±0.12	3.51±0.13	3.79±0.11	3.75±0.10

Table 39a. Larval survival (%) at PL1 stages of *P. indicus* fed two algal feeds plus five *Artemia* ml^{-1} between PZ3/M1 and PL stages. Each value represents a mean ± s.d. (n=2). (Data for Figure 4a).

Larval survival (%) ± s.d. (PL1)				
Algae/Cell concent.	50 cells μl^{-1}	60 cells μl^{-1}	70 cells μl^{-1}	80 cells μl^{-1}
T. chuii /S. costatum	51.75±2.48	69.00±1.41	67.00±6.36	58.50±4.95
S. costatum	38.75±0.35	41.25±3.18	76.50±3.54	27.75±1.79

Table 39b. Larval growth (mm TL) at PL1 stages of *P. indicus* fed two algal feeds plus five *Artemia* ml^{-1} between PZ3/M1 and PL stages. Each value represents a mean ± s.d. (n=2). (Data for Figure 4b).

Larval growth (mm) ± s.d. (PL1)				
Algae/Cell concent.	50 cells μl^{-1}	60 cells μl^{-1}	70 cells μl^{-1}	80 cells μl^{-1}
T. chuii /S. costatum	6.07±0.16	6.10±0.13	6.33±0.19	6.28±0.18
S. costatum	5.65±0.11	5.87±0.15	5.82±0.14	6.16±0.14

Experiment 3

This experiment was performed to determine whether there is any advantage in feeding *P. indicus* larvae on algae together with an animal prey during mysis and early PL stages. Figures 5a, b show larval survival and growth of *P. indicus* fed three different feeding regimes from PZ3/M1 to PL stages. Two-way analyses of variance on growth and survivals (Tables 40a and 41a) show that slope of at least one treatment was significantly different to the average slope ($P<0.001$) (See Figures 5a, b). Comparison of growth and mortality rates (slope of the treatments for survival) indicate that larvae fed algae from PZ3/M1 to PL1 stages without *Artemia* display the lowest daily growth rate (0.32 mm day^{-1}) and highest mortality rate (6.4 % day^{-1}) (Tables 40b, 41b). The growth rate (0.70 mm day^{-1}) of algae/ *Artemia* fed larvae was not significantly different to that (0.71 mm day^{-1}) of larvae fed *Artemia* alone. In addition, larvae reared on an algae/*Artemia* combination exhibited a higher mortality rate (2.07 % day^{-1}) than larvae fed solely on *Artemia* (0.67 % day^{-1}). Final survivals of algae, algae/*Artemia* and *Artemia* fed larvae were 74 %, 92 % and 98 % respectively (Table 42). Final larval total lengths were 5.37 mm (algae), 6.64 mm (algae/*Artemia*) and 6.72 mm (*Artemia*) at PL1/2 stages. Larvae fed on *Artemia* and algae/ *Artemia* started to metamorphose into PL1 stage on day 8 whereas larvae fed on algae alone only reached this stage 2 days later on day 10.

Figure 5a

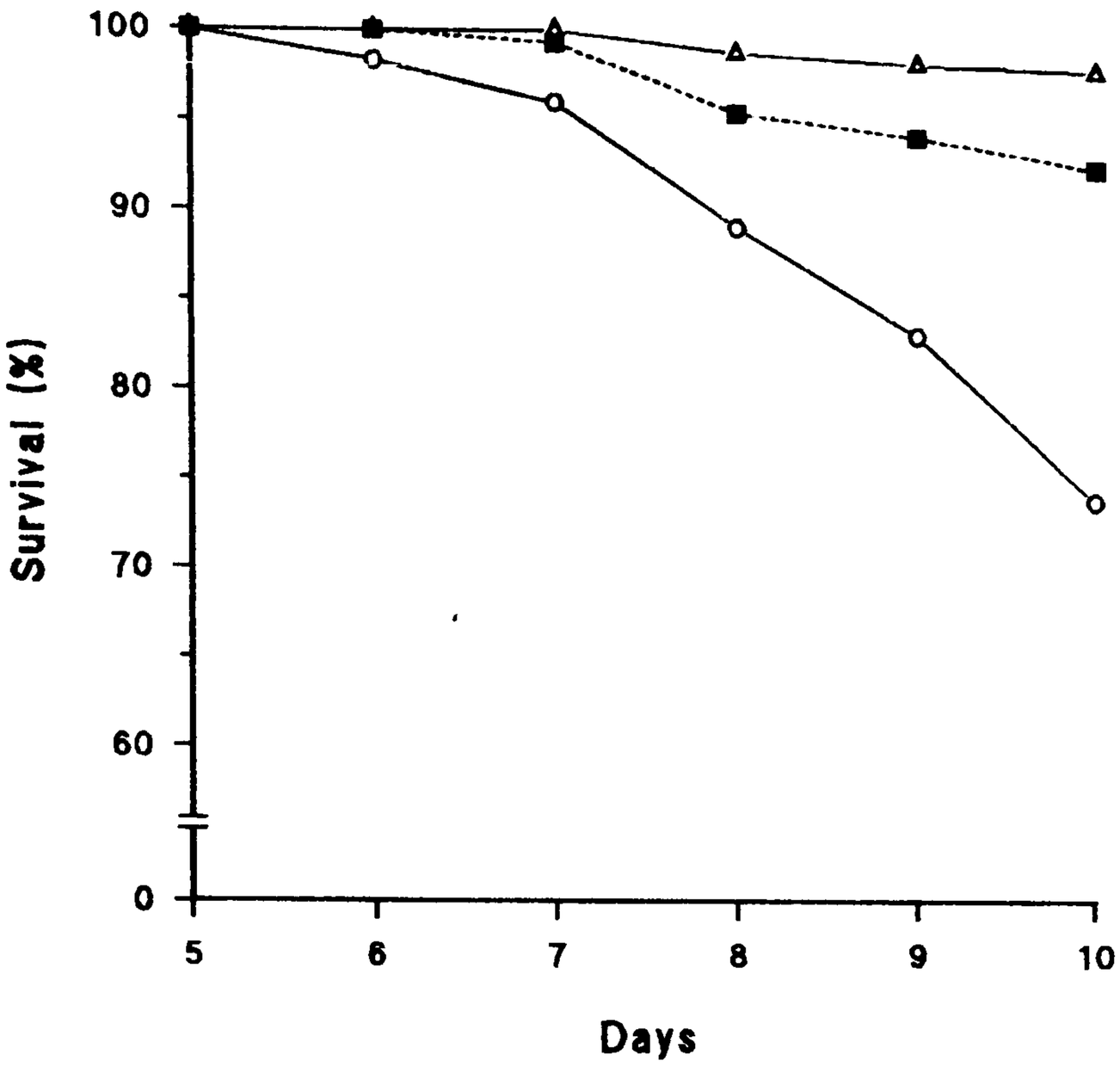
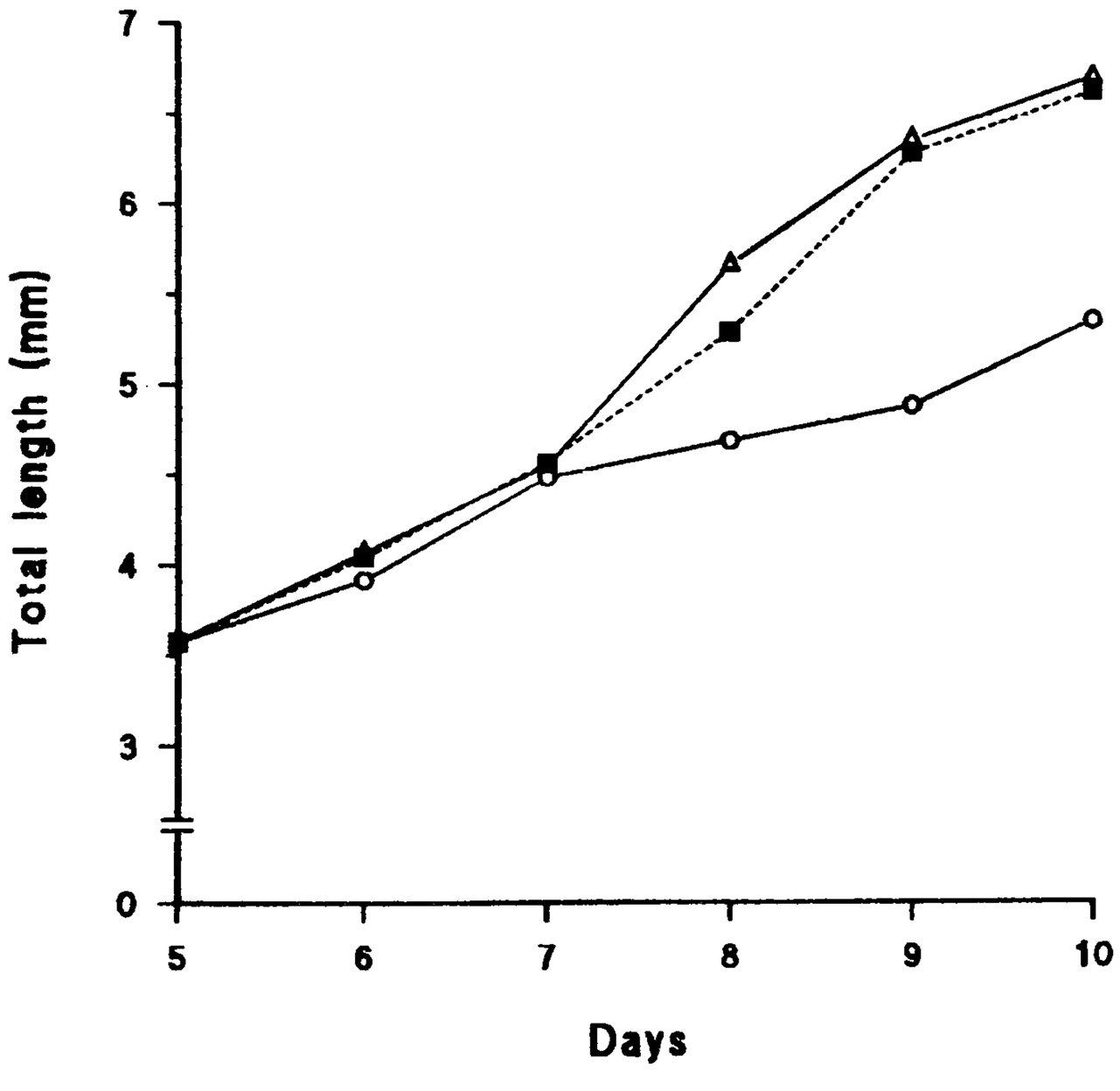


Figure 5b



○ Algae ■ Algae/Artemia ▲ Artemia

Figure 5. (a) Larval survival (%) and (b) growth (mm TL) of *P. indicus* reared on three feeding regimes, *Artemia* only, mixed algae (*T. chuii* / *S. costatum*) plus *Artemia*, and the mixed algae only from PZ3/M1 to PL1/2 stages. Each point represents a mean ± s.d. (n=2). Each replicate contains measurements of 10-13 larvae for growth.

(***) Denotes pairs of groups significantly different ($P \leq 0.001$).
(**) Denotes pairs of groups significantly different ($P \leq 0.01$).
(*) Denotes pairs of groups significantly different ($P \leq 0.05$).
(Ns) Indicates a non-significant difference ($P>0.05$).

Table 40a. Two-way ANOVA with days as covariate on larval survival by three different feeding regimes from PZ3/M1 to PL stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Diets	2	587.52	45.78	22.89	6.45	0.008	
Days	1	556.14	556.14	556.14	156.68	0.000	
repl(Diets)	3	4.45	22.99	7.66	2.16	0.128	
Diets*Days	2	357.37	357.37	178.68	50.34	0.000	***
repl*Days(Diets)	3	32.44	32.44	10.81	3.05	0.055	
Error	18	63.89	63.89	3.55			
Total	29	1601.81					

Table 40b. Comparison of larval mortality rates by three different feeding regimes from PZ3/M1 to PL.

Term	Mortality rate (% day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
Constant		103.634	0.807	128.47	0.000	
Days		-3.0445	0.2432	-12.52	0.000	
Days*Diets						
Algae	6.400	-3.3555	0.3440	-9.76	0.000	***
Algae/*Artemia*	2.067	0.9780	0.3440	2.84	0.011	*
Artemia	0.667	2.3775	0.3440	6.91	0.000	***

Table 41a. Two-way ANOVA with days as covariate on larval growth by three different feeding regimes from PZ3/M1 to PL stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Diets	2	44.342	2.514	1.257	5.76	0.003	
Days	1	224.474	225.169	225.169	1031.19	0.000	
Repl(Diets)	3	1.401	0.028	0.009	0.04	0.988	
Diets*Days	2	22.690	22.655	11.328	51.88	0.000	***
Repl*Days(Diets)	3	0.196	0.196	0.065	0.30	0.826	
Error	326	71.185	71.185	0.218			
Total	337	364.287					

Table 41b. Comparison of larval growth rates by three feeding regimes.

Term	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
Constant		3.45401	0.05984	57.72	0.000	
Days		0.57745	0.01798	32.11	0.000	
Days*Diets						
Algae	0.319	-0.25852	0.02538	-10.19	0.000	***
Algae/*Artemia*	0.704	0.12610	0.02535	4.97	0.000	***
Artemia	0.710	0.13242	0.02556	5.18	0.000	***

Table 42. Larval survival and growth of *P. indicus* by three feeding regimes from PZ3/M1 to PL stages. (Data for Figure 5a, b).

	Larval survival (%) ± s.d.			Larval growth (mm TL) ± s.d.		
Days	Algae	Algae/ *Artemia*	*Artemia*	Algae	Algae/*Artemia*	*Artemia*
5	100.00	100.00	100.00	3.575±0.059	3.575±0.059	3.575±0.059
6	98.34±2.35	100.00	100.00	3.916±0.098	4.042±0.006	4.070±0.123
7	96.00±2.83	99.34±0.95	100.00	4.489±0.047	4.569±0.098	4.549±0.070
8	89.00±0.47	95.34±0.95	98.67±1.89	4.693±0.178	5.296±0.193	5.668±0.236
9	83.00±1.41	94.00±0.95	98.00±0.95	4.891±0.058	6.295±0.108	6.368±0.167
10	73.84±4.01	92.34±2.35	97.67±2.35	5.366±0.222	6.639±0.090	6.721±0.002

Experiment 4

Following determination of the best algal feeding regime in the previous experiments, the effect of salinity on growth and survival during larval development of *P. indicus* was also investigated between PZ1 and PL stages in this experiment. Figures 6a, b show that although highest larval growth rate occurred at 25 ppt (0.68 mm TL day^{-1}), two-way analysis of variance on larval growth with days as a covariate (Table 44a) indicated that there was no significant difference between growth rates of larvae reared at salinities from 20 to 35 ppt ($P>0.05$). However, a significant difference in the salinity effect on larval mortality rate (Table 43a) was found between PZ1 and PL1 stages ($P<0.001$). Lowest mortality rates (1.65-1.80 % day^{-1}) were achieved at lower salinities (20 and 25 ppt) whereas the highest mortality rate (5 % day^{-1}) was found for larvae reared at 35 ppt S. Larvae reared at 30 ppt displayed an intermediate mortality rate (2.49 % day^{-1}).

Larvae cultured at 20 and 25 ppt had higher survivals (92-96.5 %), greater total lengths (4.01-4.03 mm at PZ3/M1) and faster development (29-32 % M1) than larvae at 30-35 ppt which sustained 87.5-93 % survival, 3.8-3.81 mm TL and slower development (4.5-18 % M1) during protozoeal stages (Figures 6a, b and Table 45). At the termination of the experiment larvae at 25 ppt exhibited 91 % survival, 6.48 mm TL compared to 69 % survival and 6.05 mm TL obtained from larvae at 35 ppt. At 25 ppt, on day 8, 32 % of the larvae were at PL2 stage whereas at 35 ppt only 5 % of the larvae were at PL2 stage (Table 45). Growth and survival data are summarised in Tables 46a, b.

Figure 6a

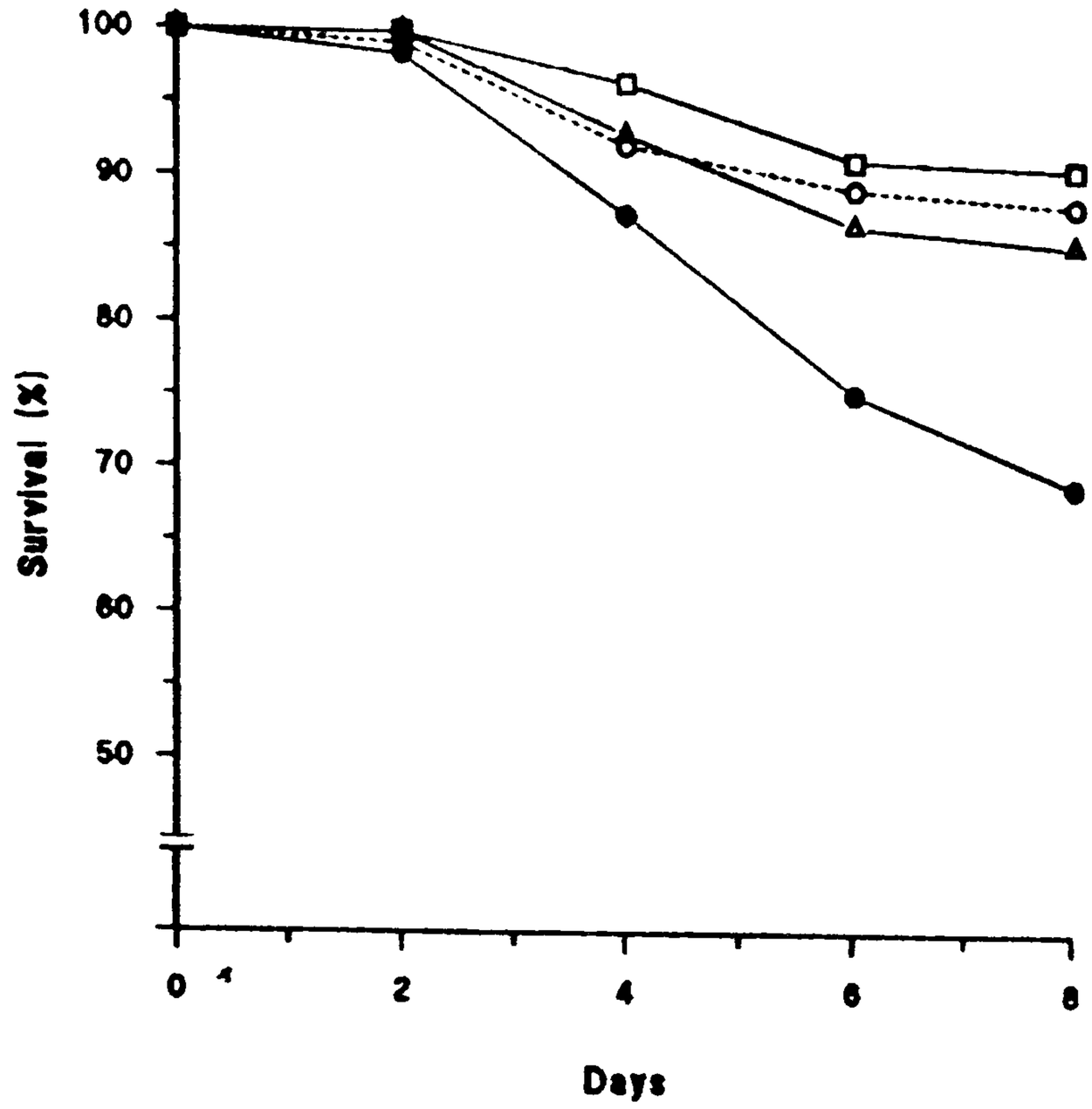
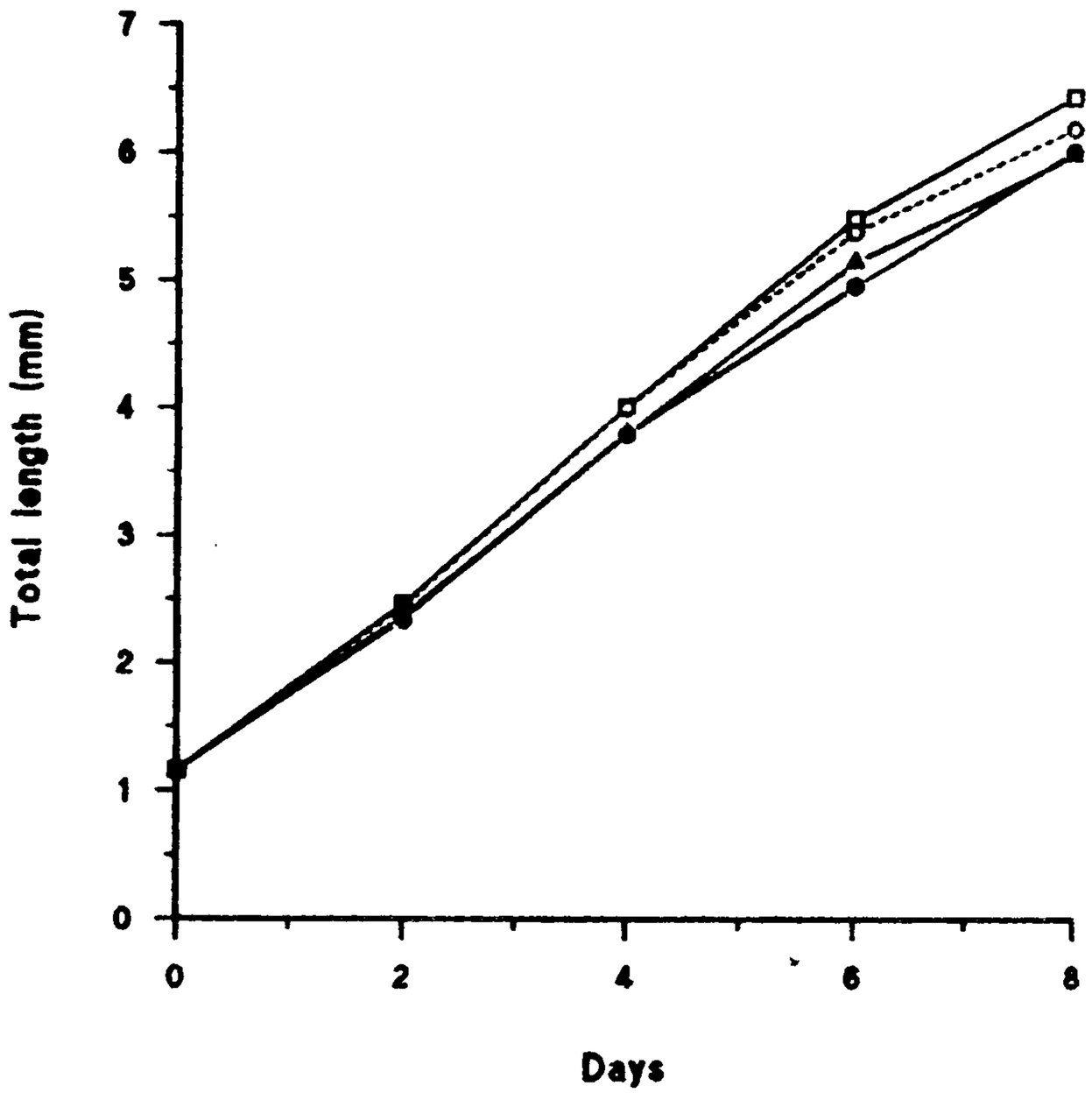


Figure 6b



○ 20 ppt □ 25 ppt ▲ 30 ppt ● 35 ppt

Figure 6. (a) Larval survival (%) and (b) growth (mm TL) of *P. indicus* cultured on live mixed algae from PZ1 to PZ3/M1 and plus *Artemia* during mysis and postlarval stages at four different salinities (20-35 ppt S). Each point represents a mean ± s.d. (n=2). Each replicate contains measurements of 10-13 larvae for growth.

(***) Denotes pairs of groups significantly different ($P \leq 0.001$).
(**) Denotes pairs of groups significantly different ($P \leq 0.01$).
(*) Denotes pairs of groups significantly different ($P \leq 0.05$).
(Ns) Indicates a non-significant difference ($P>0.05$).

Table 43a. Two-way ANOVA with days as covariate on larval survival of *P. indicus* reared in four different salinities from PZ1 to PL stages (day 2 - day 8).

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Salinity	3	666.81	30.56	10.19	1.93	0.165	
Days	1	1204.51	1204.51	1204.5	228.75	0.000	
Repl(Salinity)	4	1.94	0.44	0.11	0.02	0.999	
Salinity*Days	3	296.56	296.56	98.85	18.77	0.000	***
Repl*Days(Salinity)	4	0.81	0.81	0.20	0.04	0.997	
Error	16	84.25	84.25	5.27			
Total	31	2254.87					

Table 43b. Comparison of larval mortality rates of *P. indicus* cultured in four different salinities from PZ1 to PL stages.

Term	Mortality rate (% day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
Constant		103.906	0.994	104.57	0.000	
Days		-5.4875	0.3628	-15.12	0.000	
Days*Diets						
20 ppt	1.80	1.8875	0.6284	3.00	0.008	***
25 ppt	1.65	2.1875	0.6284	3.48	0.003	***
30 ppt	2.49	0.5125	0.6284	0.82	0.427	Ns
35 ppt	5.04	-4.5875	0.6284	-7.30	0.000	***

Table 44a. Two-way ANOVA with days as covariate on larval growth of *P. indicus* reared in four different salinities from PZ1 to PL stages (day 2 - day 8).

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Salinity	3	2.498	0.611	0.204	0.91	0.437	
Days	1	706.837	706.939	706.94	3156.41	0.000	
Repl(Salinity)	4	0.615	0.354	0.089	0.40	0.812	
Salinity*Days	3	0.775	0.772	0.257	1.15	0.329	Ns
Repl*Days(Salinity)	4	0.378	0.378	0.094	0.42	0.793	
Error	332	74.358	74.358	0.224			
Total	347	785.461					

Table 44b. Comparison of larval growth rate (mm day^{-1}) of *P. indicus* reared at four different salinities.

Term	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
Constant		1.25317	0.06330	19.80	0.000	
Days		1.28964	0.02295	56.18	0.000	
Days*Salinity						
20 ppt	0.627	-0.03555	0.03947	-0.90	0.368	Ns
25 ppt	0.682	0.07294	0.03990	1.83	0.068	Ns
30 ppt	0.637	-0.01571	0.03970	-0.40	0.693	Ns
35 ppt	0.634	-0.02169	0.03997	-0.54	0.588	Ns

Table 45. Composition of larval stages of *P. indicus* cultured at various salinities.

	Larval composition (%)			
Days / Salinity	20 ppt	25 ppt	30 ppt	35 ppt
Day 4 (PZ3/M1)	29 % M1, 71 % PZ3	32 % M1, 68 % PZ3	18 % M1, 82 % PZ3	4.5 % M1, 95.5 % PZ3
Day 8 (PL1/2)	68 % PL1, 32 % PL2	68 % PL1, 32 % PL2	91 % PL1, 9 % PL2	95 % PL1, 5 % PL2

Table 46a. Larval survival (%) of *P. indicus* cultured at four different salinities from PZ1 to PL stages. Each value is a mean ± s.d. (n=2). (Data for Figure 6a).

	Larval survival (%) ± s.d.			
Days	20 ppt	25 ppt	30 ppt	40 ppt
0	100.00	100.00	100.00	100.00
2	99.25±0.35	100.00	100.00	98.50±2.12
4	92.25±1.06	96.50±0.71	93.00±0.00	87.50±2.12
6	89.25±0.35	91.25±1.06	86.75±1.77	75.25±1.74
8	88.25±1.06	90.75±1.77	85.50±0.71	69.00±1.41

Table 46b. Total length (mm) of *P. indicus* cultured at four different salinities from PZ1 to PL stages. Each value is a mean ± s.d. (n=2). (Data for Figure 6b).

	Larval total length (mm) ± s.d.			
Days	20 ppt	25 ppt	30 ppt	40 ppt
0	1.16±0.05	1.16±0.05	1.16±0.05	1.16±0.05
2	2.45±0.04	2.47±0.09	2.37±0.02	2.34±0.04
4	4.01±0.21	4.03±0.04	3.81±0.08	3.80±0.02
6	5.41±0.06	5.51±0.01	5.17±0.01	4.98±0.05
8	6.23±0.04	6.48±0.01	6.02±0.07	6.05±0.20

Experiment 5

Figures 7a and 7b show growth and survival of *P. indicus* larvae fed *R. reticulata* at 50 cells μl^{-1} compared with those fed control mixed algal feed (*T. chuii /S. costatum*) at 60 cells μl^{-1} from PZ1 to PZ3/M1 stages. From the first day, larvae fed *R. reticulata* exhibited lower survival and growth compared to the mixed algae fed ones. Comparison of larval survival between day 2 and day 6 (Tables 48a, b) demonstrated that there was a significant ($P<0.001$) difference between survival of the larvae fed the two algal feeding regimes during protozoeal stages. Larvae fed *R. reticulata* showed significantly higher mortality rate (20.4 % day^{-1}) than those fed the mixed algae (7.43 % day^{-1}). Hence, larval survival on the mixed algae and *R. reticulata* on day 6 were 73% and 15% respectively (see Table 47). Figure 7b and Tables 49a, b show that larval growth rate on the two algal feed regimes were also significantly ($P<0.001$) different. Larvae fed the mixed algae grew 0.75 mm TL day^{-1} whereas those fed the single alga had a total length increment of only 0.38 mm day^{-1}. At the termination of the experiment (on day 6) final lengths of the larvae fed the mixed algae and *R. reticulata* were 4.26 mm and 2.87 mm respectively. On day 6, larval stages on the single alga were 95 % PZ3 and 5 % PZ2. All the larvae on the mixed algae were, however, at M1 stage. Starved larvae (PZ1) completely perished 24 h after they were stocked into the experimental flasks.

Observations of larval guts and faeces of the larvae showed that the larvae ingested and digested the cells of *R. reticulata*. When PZ1, PZ2 or PZ3 larvae on *R. reticulata* were sampled, the guts full of the red algae were clearly visible. No undigested cells were observed in larval faeces at any stage during protozoeal stages. The cells of *R. reticulata*, in control flasks without larvae, were slightly increased from 50 cells μl^{-1} to 57 cells μl^{-1} over 24 h.

Figure 7a

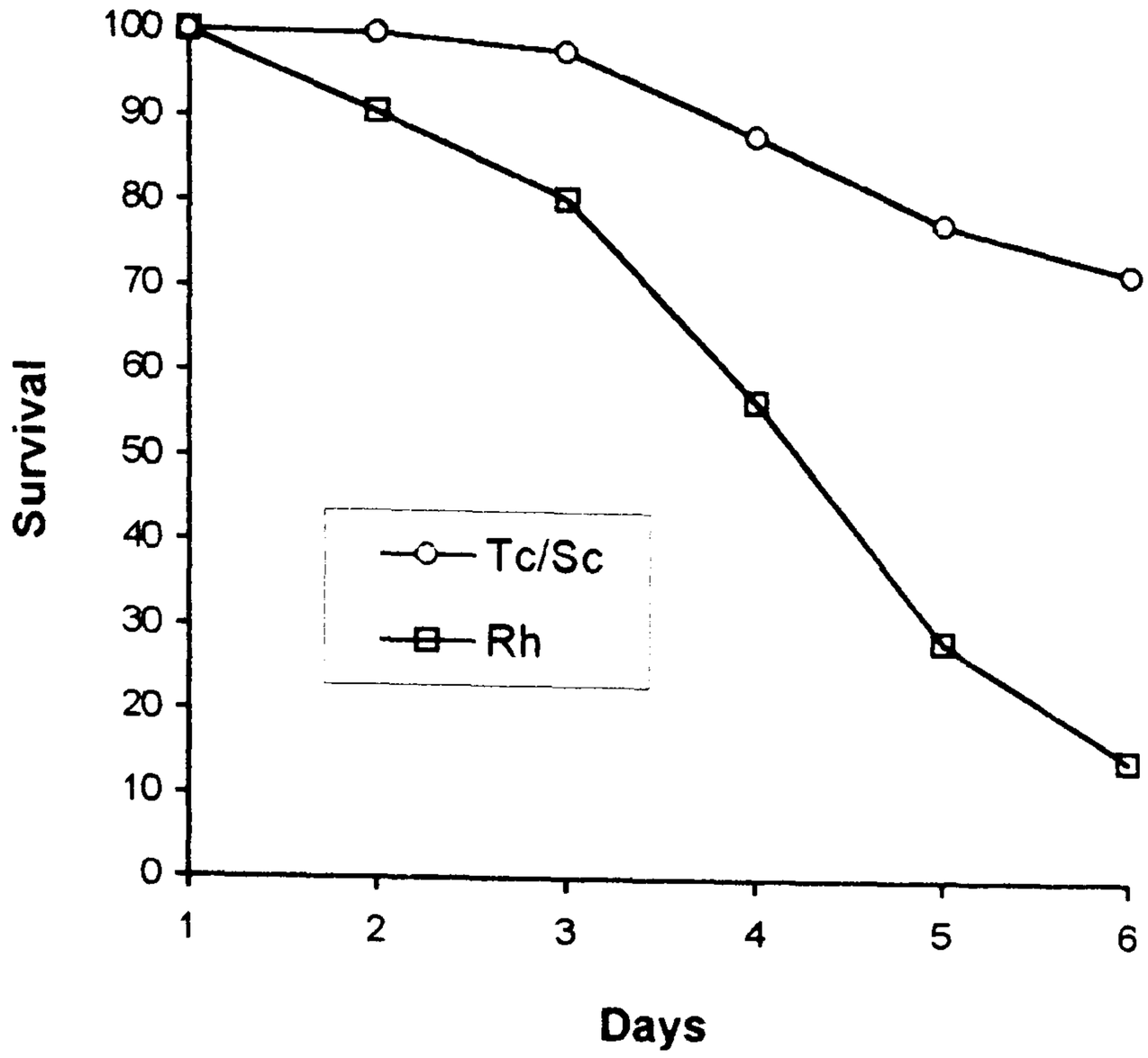


Figure 7b

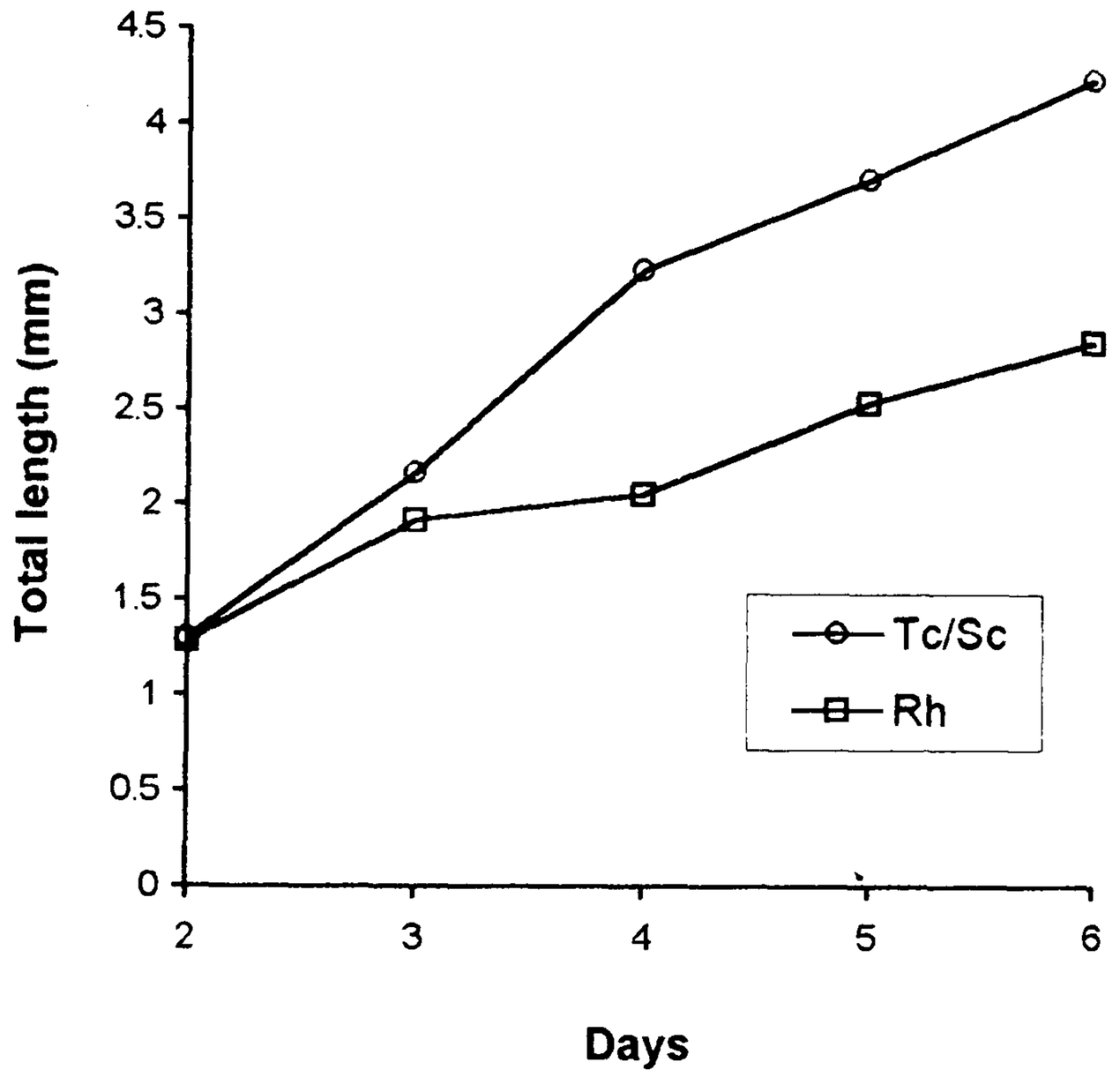


Figure 7. (a) Larval survival (%) and (b) growth (mm TL) of *P. indicus* cultured on live mixed algae (*T. chuii* / *S. costatum*=Tc/Sc) and *R. reticulata* (Rh) from PZ1 to PZ3/ M1. Each point represents a mean ± s.d. (n=2). Each replicate contains measurements of 10-13 larvae for growth.

(***) Denotes pairs of groups significantly different ($P \leq 0.001$).
(**) Denotes pairs of groups significantly different ($P \leq 0.01$).
(*) Denotes pairs of groups significantly different ($P \leq 0.05$).
(Ns) Indicates a non-significant difference ($P>0.05$).

Table 47. Survival and growth of *P. indicus* larvae fed two feeding regimes from PZ1 to PZ3/M1 stages. (Tc/Sc= *T. chuii/ S. costatum* and Rh= *R. reticulata*).

	Survival (%) ± s.d.		Total length (mm) ± s.d.	
Day	Tc/Sc	Rh	Tc/Sc	Rh
1	100.00	100.00	0.97 ± 0.01	0.97 ± 0.01
2	100.00	90.67 ± 9.43	1.30 ± 0.02	1.28 ± 0.02
3	98.00 ± 2.83	80.67 ± 11.31	2.16 ± 0.07	1.92 ± 0.10
4	88.34 ± 8.01	57.00 ± 6.12	3.24 ± 0.19	2.06 ± 0.02
5	78.33 ± 12.73	28.67 ± 16.97	3.72 ± 0.12	2.54 ± 0.09
6	72.67 ± 10.37	14.67 ± 8.49	4.26 ± 0.08	2.87 ± 0.02

Table 48a. Two-way ANOVA with days as a covariate on larval survival from PZ1 to PZ3/M1 stages on two feeding regimes.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Diets	1	5488.3	30.2	30.2	0.31	0.586	
Days	1	7746.8	7746.8	7746.8	80.34	0.000	
Days(Diets)	2	1.6	11.6	5.8	0.06	0.942	
Diets*Days	1	1680.9	1680.9	1680.9	17.43	0.001	***
Repl*Days(Diets)	2	17.4	17.4	8.7	0.09	0.914	
Error	12	1157.1	1157.1	96.4			
Total	19	16092.2					

Table 48b. Comparison of mortality rates of the larvae fed two feeding regimes from PZ1 to PZ3/M1 stages.

Term	Mortality rate (% day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
Constant		112.650	5.149	21.88	0.000	
Days		-13.917	1.553	-8.96	0.000	
Days*Repl						
Tc/Sc	7.43	6.483	1.553	4.18	0.001	***
Rh	20.4	-6.483	1.553	-4.18	0.001	***

Table 49a Two-way ANOVA with days as a covariate on larval growth from PZ1 to PZ3/M1stages on two feeding regimes (Tc/Sc= *T. chuii/S. costatum* and Rh= *R. reticulata*).

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Diets	1	30.801	0.958	0.958	12.18	0.001	
Days	1	135.078	132.717	132.717	1687.19	0.000	
Repl(Diets)	2	0.003	0.037	0.019	0.24	0.789	
Diets*Days	1	14.316	14.328	14.328	182.14	0.000	***
Repl*Days(Diets)	2	0.051	0.051	0.026	0.33	0.723	
Error	205	16.126	16.126	0.079			
Total	212	196.375					

Table 49b. Comparison of growth rates of the larvae on two feeding regimes from PZ1 to PZ3/M1 stages.

Term	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
Constant		0.84104	0.045	18.64	0.000	
Days		0.56467	0.014	41.08	0.000	
Days*Repl						
Tc/Sc	0.750	0.18553	0.014	13.50	0.000	***
Rh	0.379	-0.18553	0.014	-13.50	0.000	***

DISCUSSION

The present data indicate that low cell concentrations of either single algal feeds or mixed algal diets do not give satisfactory larval growth and survival in *P. indicus*. 10-20 cells μl^{-1} of flagellate *T. chuii* and diatom *S. costatum* fed individually did not support survival further than the PZ1/ PZ2 stages suggesting that this penaeid species cannot be reared successfully with low cell densities using the present rearing methods. However, when the mixed algal (*T. chuii /S. costatum*) diet was fed at 20 cells μl^{-1}, 28 % survival was obtained (Table 3a) at PZ1-PZ3/M1 stages. Emmerson (1980) reports very good survival (96 %) and normal larval development using a cell density of *T. weissflogii* (10.7 µm in diameter) at 7 cells μl^{-1} for the culture of *P. indicus* at a density of 35 larvae l^{-1} in large (70-l) culture vessels at 26 °C. This author darkened the larval culture room to prevent cell increase as a result of high light intensity. In the present study, however, experiments were all conducted under continuous light conditions (2.56×10^{14} quanta /sec / cm ^{2}) provided by fluorescent light bulbs. It appears that the present higher larval stocking density (100 larvae l^{-1}) produces a grazing demand which cannot be met at low cell densities. Amjad (1990) reports total mortality of *P. monodon* larvae at a cell density of 10 cells μl^{-1} and low survival at 20 cells μl^{-1} (*T. chuii / R. reticulata*) under similar culture conditions to the present experiments. Aquacop (1983) suggests that a cell density of 100 cells μl^{-1} of mixed algae is required at high larval stocking densities (100-120 larvae l^{-1}), whereas Galgani and Aquacop (1988) recommend 30-40 cells μl^{-1} algae at 100 l^{-1} larval stocking density during protozoeal culture of *P. indicus*.

As cell density was increased from 30 to 40 and 50 cells μl^{-1}, larval survival and growth progressively increased (Figures 1a, b). This indicates that low algal cell density cannot provide sufficient nutrient/ energy at 100 larvae l^{-1} for *P. indicus*. Kurmaly et al., (1989b) suggest that *P. monodon* PZ1 larvae

at this stocking density require 45 cells μl^{-1} day^{-1} for optimal survival and growth. In present experiments this level is reached at 60-70 cells μl^{-1} day^{-1}, hence levels below these are inadequate for larval growth, survival and development. Figures 1a, b and Tables 3a, b exhibit progressively increased growth from 2.58 mm TL up to 4.08 mm TL and survivals from 28 % to 80 % when *P. indicus* larvae were fed on increased algal cell densities from 20 cells μl^{-1} to 50 cells μl^{-1} (*T. chuii* / *S. costatum*) at PZ1-PZ3/M1 stages. This pattern was found to be the same for the larvae reared on single algal feeds. For example, when larvae were fed 30 cells μl^{-1} of *S. costatum*, 14 % survival and 2.59 mm TL was obtained at PZ3/M1 stages, whereas larvae fed 50 cells μl^{-1} gave 50 % survival and 3.92 mm TL. Low survival and growth obtained during protozoeal stages affected the subsequent results at metamorphosis despite the fact that the larvae were fed on *Artemia* nauplii from PZ3/M1 onwards (Figures 2a, b).

Jones and Kurmaly (1987) report that *P. monodon* larvae refill their guts 5-7 times h^{-1} when feeding on dense algal cell concentrations. Assimilation efficiency of this species was found to be low at a high cell density (60 cells μl^{-1}), but optimal energy gains may be obtained when the food is shunted rapidly through the gut and only partially digested but replenished quickly (Kurmaly et al., 1989b). The best larval growth and survivals in the present investigation were obtained at a cell density of 60-70 cells μl^{-1} on the mixed algal feed *T. chuii* / *S. costatum* (Figures 3-4). The diatom *S. costatum* supported higher survival and growth at 70-80 cells μl^{-1} during protozoeal stages. With the addition of *Artemia* during mysis stages, however, at a cell density of 80 cells μl^{-1}, this alga aggregated on the cephalic appendages of the larvae and caused high mortality.

Different growth and survival responses of penaeid larvae to the algal feeds may be due to variations in the nutritive value, cell size, digestibility or chemical composition of algal species used (Tobias-Quinitio and Villegas,

1982; Volkman et al., 1989). In the present study, it is unlikely that cell size of the algal feeds is inappropriate for the larvae (Table 1). Penaeid species are generally fed on food particles ranging from 10-28 µm (Jones et al., 1979a) to 75 µm (Hirata et al., 1975). Moreover, *P. indicus* larvae are capable of employing both filter and raptorial feeding strategies as early as PZ1/PZ2 (Emmerson, 1980; see Chapter 3) as are other penaeid species such as *P. kerathurus* (Yúfera et al., 1984), *P. marginatus* (Gopalakrishnan, 1976), *P. vannamei* and *P. setiferus* (Wilkenfeld et al, 1984). Galgani and Aquacop (1988) report that PZ1 and PZ2 stages of *P. indicus* are capable of taking food particles of up to 35-50 µm.

Evaluation of algae as live feeds for penaeid larvae is generally based on selection of species that sustain the maximum growth, survival and development. The present study suggests that for the three unicellular algal species tested, the diatom *S. costatum* promotes better larval growth, survival and development throughout all larval stages than the flagellate *T. chuii* (Tobias-Quinitio and Villegas, 1982). It is known that *S. costatum* is one of the most suitable live diets for penaeid larvae during protozoeal stages (Preston, 1985a) and therefore is commonly used in hatcheries (Jones et al., 1987). In the second experiment, larvae reared solely on this algal species from PZ1 to PZ3/M1 (Figures 3a, b) displayed high survivals (88-90%) and good growth (3.75-3.79 mm TL) at 70-80 cells μl^{-1}. Kuban et al., (1985) also state that diatoms (*S. costatum* and *C. gracilis*) were better food sources than phytoflagellates for *P. vannamei* and *P. stylirostris*. However it has been observed that *S. costatum* at high cell concentrations causes mechanical fouling that may hamper feeding and respiration of penaeid larvae (Jones et al., 1987; Kurmaly et al., 1989a). Liao et al., (1983) report that exclusive use of *S. costatum* may be harmful to penaeid larvae if the alga is harvested in or after the stationary growth phase. In contrast, other investigations find flagellates such as *T. chuii* and *R. reticulata* sustain better growth and survival than *S. costatum* during protozoeal stages of *P.*

monodon (Amjad, 1990). The present results show that *R. reticulata*, as a single diet even at 50 cells μl^{-1}, did not promote good survival and growth further than PZ3/M1 stages (see Experiments 1 and 5). However, Kurmaly et al., (1989a) and Amjad (1990) report that *R. reticulata* gave higher survival and better growth for *P. monodon* than *T. chuii* and/or *S. costatum* when fed as a single alga. Based on the present results and those of Kurmaly et al., (1989a) and Amjad (1990), it is difficult to see why *R. reticulata* is a very suitable food for *P. monodon*, but is inadequate for *P. indicus*. Observations of the gut of the larva fed *R. reticulata* and their faeces under a microscope revealed that this alga was ingested and digested. In addition, this alga either alone, or with *T. chuii*, induces high larval trypsin activities when fed to *P. indicus* larvae (see Chapter 5). Results of the fifth experiment also showed that cell numbers of this algal species remain approximately constant for a period of 24 h in 2-l flasks without larvae. Moreover, when high larval mortalities were observed at PZ2/Z3 stage on *R. reticulata* at 50 cells μl^{-1}, the larval guts were always full of algae, and larval faeces were also clearly visible (Experiment 5). In preliminary trials, *R. reticulata* as a single diet (see also Experiment 1), and in combination with *T. chuii* (50-70 cells μl^{-1}, 1:1) promoted survivals of only 5.5 % and 5.5-6.5 % until PL1 stage (Kumlu, unpublished data). Heavy larval mortalities obtained using *R. reticulata* both as a single feed and in combinations with other algae in the present experiments suggest that this alga may be nutritionally inadequate for *P. indicus* larvae.

Some algal species such as *Chlorella* sp. and *Pavlova* sp. are known to be nutritionally poor for most aquatic species (Gopalakrishnan, 1976; Aujero et al., 1983; Amjad, 1990). Nutritive value of microalgae may greatly vary even within the same species depending on culture conditions and time of harvest (Léger et al., 1986). The macronutrient content and fatty acid profile of the algal species used in the present study are summarised in Tables 1 and 2. Quantities of these macronutrients in all the algal species are

within the range required by penaeid larvae (Kurmaly et al., 1989a; Kanazawa, 1990; Chen, 1993). The importance of ω3 highly unsaturated fatty acids (HUFA), especially eicosapentaenoic acid (20:5ω3) and docosahexaenoic acid (22:6ω3) have been demonstrated for marine and brackish water prawns (Jones et al., 1979a; Kanazawa et al., 1979; Guillaume, 1990). Marine prawns cannot synthesise these ω3 HUFA rapidly enough to meet their physiological requirements (Jones et al., 1979a), therefore, these are required in diets (Léger and Sorgeloos, 1992). *T. chuii* has a high protein content but a low lipid level with a low level of 20:5ω-3 and no 22:6ω-3. *S. costatum*, in contrast, contains low protein, high lipid and high levels of the essential fatty acids (see Tables 1, 2). This may explain why the diatom *S. costatum* gave better larval growth and survival than the flagellate *T. chuii*. When Rodríguez et al., (1994) fed *P. japonicus* mysis larvae on *Chaetoceros gracilis* containing only 7% protein and separately on *Artemia* with a much higher protein content, they obtained no significant difference in growth, survival, protein or lipid content in postlarvae. In the present study, the combination of *T. chuii* / *S. costatum* consistently gave significantly superior larval growth and survival than single algal feeds ($P<0.05$) both in the first and second experiments. This confirms that *P. indicus* larvae reared on more than one algal species with a wide diversity of macronutrients and micronutrients such as vitamins, have a better chance of meeting nutritional requirements (Amjad, 1990). The present results demonstrate that penaeid larvae show species specific differences between algae and this confirms the advantage of using mixed algal species to ensure good results (Galgani and Aquacop, 1988; Kurmaly et al., 1989a; Amjad, 1990).

Data from the third experiment suggests that there may not be any benefit to *P. indicus* larvae by feeding algal diets during mysis stages as *Artemia* only fed larvae from PZ3/M1 to PL stages displayed equal growth rates and better survival rates to those fed on algae and *Artemia* (Figures 5a, b). Larvae fed mixed algae without the animal prey showed significantly higher

mortality (6.4 % day^{-1}) and slower growth rate (0.32 mm TL day^{-1}) in comparison to algae/*Artemia* (2.07% day^{-1}, 0.70 mm TL day^{-1}) and *Artemia* only fed larvae (0.67 % day^{-1}, 0.71 mm TL) throughout mysis stages to metamorphosis (Tables 40b, 41b). Studies with other penaeid larvae such as *P. marginatus* (Gopalakrishnan, 1976), *P. aztecus*, *P. setiferus* and *P. vannamei* (Kuban et al., 1985) have also shown that exclusive use of algae throughout all larval stages results in lower growth and delayed metamorphosis although comparable survival rates can be achieved. Rodríguez et al., (1994) obtained significantly higher growth and survival when they fed *P. japonicus* larvae on alga (*C. gracilis*) throughout larval stages together with *Artemia* during mysis stages as opposed to alga only fed larvae. In their study, survival and growth of larvae receiving either alga or *Artemia* as sole diets during mysis stages did not differ significantly. In the present study, when PZ3/M1 stages of *P. indicus* were fed on *Artemia* as a sole diet, 98 % of them metamorphosed into PL1/2 stages with a final total length of 6.72 mm compared to 92 % survival and 6.64 mm TL when fed on algae /*Artemia*. Algae as a sole diet promoted 74 % survival and 5.37 mm TL when the experiment was terminated (Table 42). Again it would appear that variation in nutritional content of live feeds may explain differences in results.

Whilst growth rates of larvae over the range of test salinities did not differ significantly ($P>0.05$), survival rate and larval development (see Table 45) both during protozoeal stages and mysis stages were significantly affected by the salinity ($P<0.01$) indicating that *P. indicus* prefers lower salinities for larval development. Preston (1985a) suggests that optimal salinity for larval growth and survival depends on salinity conditions operating where spawning takes place and that salinity has more pronounced effects on protozoeal rather than mysis stages of *Metapenaeus bennettae*. In the present study although 35 ppt salinity was closest to the spawning salinity (33.5 ppt S), highest larval mortality and a lower growth rate were obtained at

this salinity. At optimum salinity, which appears to be between 20 ppt and 25 ppt, with the mixed algae (*T. chuii* / *S. costatum*) and *Artemia* from PZ3/M1 stage onwards, over 91% larvae metamorphosed into PL1/2 stages with a final total length of 6.48 mm within 8 days (Figures 6a, b). Bukhari et al., (1993) report that 30 ppt salinity was the best salinity for *P. indicus* larvae obtained from the spawners kept in sea water at 43 ppt S (Red Sea). Data from Chapter 2 also shows that optimal salinity, which sustains the maximum survival and growth during nursery stages of *P. indicus* postlarvae, is also 25 ppt.

Gopalakrishnan (1976) states that none of newly hatched larvae of *P. marginatus* were able to survive further than a few hours at 20 ppt S and that the lowest salinity tolerated by the larvae (PZ1 to PL stages) lies between 24 and 26 ppt S. This author found the highest survival of *P. marginatus* at 33 ppt S. The complete mortality obtained by Gopalakrishnan (1976) at 20-26 ppt S for nauplius and protozoeal stages could be due to abrupt salinity change to these low experimental salinities. In the present study PZ1 larvae were acclimatised to experimental salinities for 2 h (Preston 1985b). Since, heavy larval mortalities of *M. bennettae*, *P. plebejus* and *M. macleayi* at 10 and 50 ppt S were reported (Preston, 1985b), a narrower salinity range of between 20 and 35 ppt was tested on growth and survival on *P. indicus* in the current study.

The present study demonstrates that optimal larval culture conditions for *Penaeus indicus* from India at which they can be reared from PZ1 to PL1 within 6 days with over 90% survival are in water at 25 ppt salinity and at 27-28 °C using a mixed algal diet of *Tetraselmis chuii* (25 cells μl^{-1}) and *Skeletonema costatum* (35-45 cells μl^{-1}) plus five *Artemia* ml^{-1} after PZ3/M1 stage.

CHAPTER 2

SALINITY TOLERANCE OF HATCHERY REARED POSTLARVAE OF *Penaeus indicus* ORIGINATING FROM INDIA.

This chapter has been contributed to the following publication:

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Authors: Kumlu, M. and Jones, D. A.

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INTRODUCTION

The life cycle of shrimps included in the family Penaeidae is well known with most species spending their nursery stages in shallow inshore, brackish waters and moving offshore for maturation and spawning at oceanic salinities. Among ecological factors, salinity is one of the most important environmental factors affecting the growth and survival of penaeids, particularly in nursery areas where the animals may be exposed to rapid salinity fluctuations and extreme environmental conditions (Raj and Raj, 1982). Food consumption, conversion efficiency and, hence, growth, and survival of cultured penaeid shrimps are influenced by salinity and/or temperature (Venkataramaiah et al., 1972; Staples and Heales, 1991). Thus, it is important to determine optimum salinity levels for each commercial shrimp species when the postlarvae are reared in closed systems during the nursery stages. In such systems, the salinity can be altered according to optimum requirements of the species and even for individual postlarval stages.

Several studies have been conducted to determine salinity tolerances alone or in conjunction with other abiotic factors and to measure osmoregulatory ability for commercially important penaeid species such as *P. monodon*, (Cawthorne et al., 1983); *P. semisulcatus* (Harpaz and Karplus, 1991); *P. aztecus* (Venkataramaiah et al., 1972); *P. setiferus* (Zein-Eldin and Griffith, 1969); *P. vannamei*, *P. stylirostris*, *P. californiensis*, *P. brevirostris* (Mair, 1980); *P. merguiensis*, *P. esculentus*, *Metapenaeus bennettae* (Dall, 1981); *P. japonicus* and *P. chinensis* (Charmantier-Daures et al., 1988). In contrast to adults, the postlarvae and juveniles of most of these species adapt and osmoregulate well at low salinities. Studies, with juvenile Indian white shrimp, *Penaeus indicus*, revealed that this species is capable of osmoregulating at salinities of between 3 and 40 ppt (Parado-Estepa et al., 1987; Diwan and Laximinarayana, 1989). Consequently, this species may be a good candidate for culture in brackish water ponds, which may display extreme

fluctuations in salinities during high rainfall seasons. Raj and Raj (1982) studied the effect of salinity on the growth and survival of wild *P. indicus* postlarvae and juveniles and reported superior growth and survival for this species at low salinities (5-25 ppt S) following a 7 days acclimation to experimental salinities. In contrast, Bukhari et al., (1994), and Bukhari (1994) report that hatchery reared *P. indicus* postlarvae (PL1-PL60) from the Red Sea acclimatised for 10 days demonstrated the best survival, growth and biomass at high saline conditions (50 ppt S) in comparison to salinities of 10-40 ppt.

The primary aim of the present work was to culture *P. indicus* originating from India under similar experimental conditions to those used by Bukhari et al., (1994) for the culture of the same species from the Red Sea. The process and the length of acclimation period to different salinities may affect subsequent results (Cawthorne et al., 1983; Harpaz and Karplus, 1991). Therefore, these parameters were kept the same with those used by Bukhari et al., (1993; 1994).

MATERIALS AND METHODS

All postlarvae used in the experiments were obtained from Indian brood-stock, held in a recirculation system in the School of Ocean Sciences, Menai Bridge, UK. PLs (postlarvae) were reared from PL1 to PL7 on live *Artemia salina* nauplii and on a PL Frippak diet (INVE Aquaculture, Belgium) until PL20.

In the first experiment, PL7 postlarvae, previously reared at 30 ppt S, were stocked directly in experimental salinities at a density of 6 animals l^{-1} in 6-l plastic basins (25 x 30 x 15 cm) supplied with gentle aeration, fed Frippak PL diet and fresh mussel meal to excess. Seven different salinities (5, 10, 20, 25, 30, 35, 40 ppt S) were used to test the salinity resistance of the postlar-

vae from stage PL7 to PL22. After mass mortalities occurred at 10 and 5 ppt S, the animals were gradually acclimated for 48 h by reducing the salinity by 5 ppt S 2-3 times a day from 30 ppt to 5 ppt S. Since the postlarvae subjected to 10 ppt S showed high mortality in 24 h, they were excluded in the calculations. The results obtained from the PLs acclimated to 5 ppt over 48 h were also not considered in statistical analysis as acclimation was not used for animals transferred to other salinity regimes.

In the second experiment, postlarvae were stocked at a density of 4 individuals l^{-1} in 6-l plastic basins and acclimatised from 30 ppt S to different salinities (10, 20, 30, 35, 40, 50 ppt S) gradually (5 ppt each day) for up to 4 days. All the animals were kept in the final test salinities for 6 days before the first sampling was carried out on the 10th day. Desired salinities were obtained by mixing aquarium salt "Instant Ocean" (Aquarium Systems) and dechlorinated tap water, and salinity measurement was performed with a direct reading salinometer. PLs were fed on a Frippak formulated pelleted artificial diet from PL20 to PL60. At the end of the experiment postlarvae (PL60) were weighed on a scale (± 0.01 g) to determine individual wet-weight and total biomass.

Experiments were conducted under a controlled photoperiod (10L:14D) with artificial fluorescent illumination and the water temperature was maintained at 29-31 °C. Uneaten food was removed daily when 50 % of the rearing water was renewed. A complete water exchange was performed every 5 days, and simultaneously the postlarvae were counted and 10-12 of them were measured from the tip of the rostrum to the end of the tail using callipers (± 0.01 mm). All salinity treatments in both experiments were triplicated. These experimental conditions replicated those conducted in Saudi Arabia with Red Sea *P. indicus* which were also supervised by D. A. Jones.

Statistical Analysis

In the first experiment, growth and survival data were analysed by one-way ANOVA between treatments for each stage and between larval stages for each treatment. Tukey's test was used to compare the means. Slopes of growth and survival of postlarvae were calculated from 3 replicates and compared using the General Linear Model (GLM) in Minitab. Biomass and wet-weight of the animals reared at different salinities were also compared (3 replicates) by one-way ANOVA and Scheffe's test.

RESULTS

Experiment 1

When PL7 stage animals were transferred from 30 ppt S to 5 and 10 ppt S, mass mortalities (between 80 and 100 %) occurred within 24 h. However, following a gradual acclimatisation over a period of 48 h, the postlarvae were able to adapt successfully to 5 ppt S. Thereafter, this low salinity appeared to be one of the most favourable salinities during the early nursery stages of *P. indicus* (see Figures 1a, b). Exposure of the specimens to a salinity increase from 30 to 40 ppt S or decrease to 20 ppt S had no detrimental effect on growth and survival.

Figure 1 shows that although survival of PLs reared under different salinities for a period of one week did not differ from each other ($P>0.05$), growth at low salinities (20-30 ppt) were significantly better than high salinities (35-40 ppt) (see Tables 1-6). Animals directly transferred from 30 ppt S, reared at 25 ppt S had the highest survival (92.22 %) and the fastest growth (18.2 mm total length = TL), whereas animals at 40 ppt S showed the lowest survival (73 %) and growth (12.33 mm TL) to PL22 stage ($P<0.05$) (see Tables 3 and 6). Although the PLs had no salinity stress at 30 ppt, their survival and total length did not differ significantly from animals reared at 20 ppt S ($P>0.05$).

Figure 1a

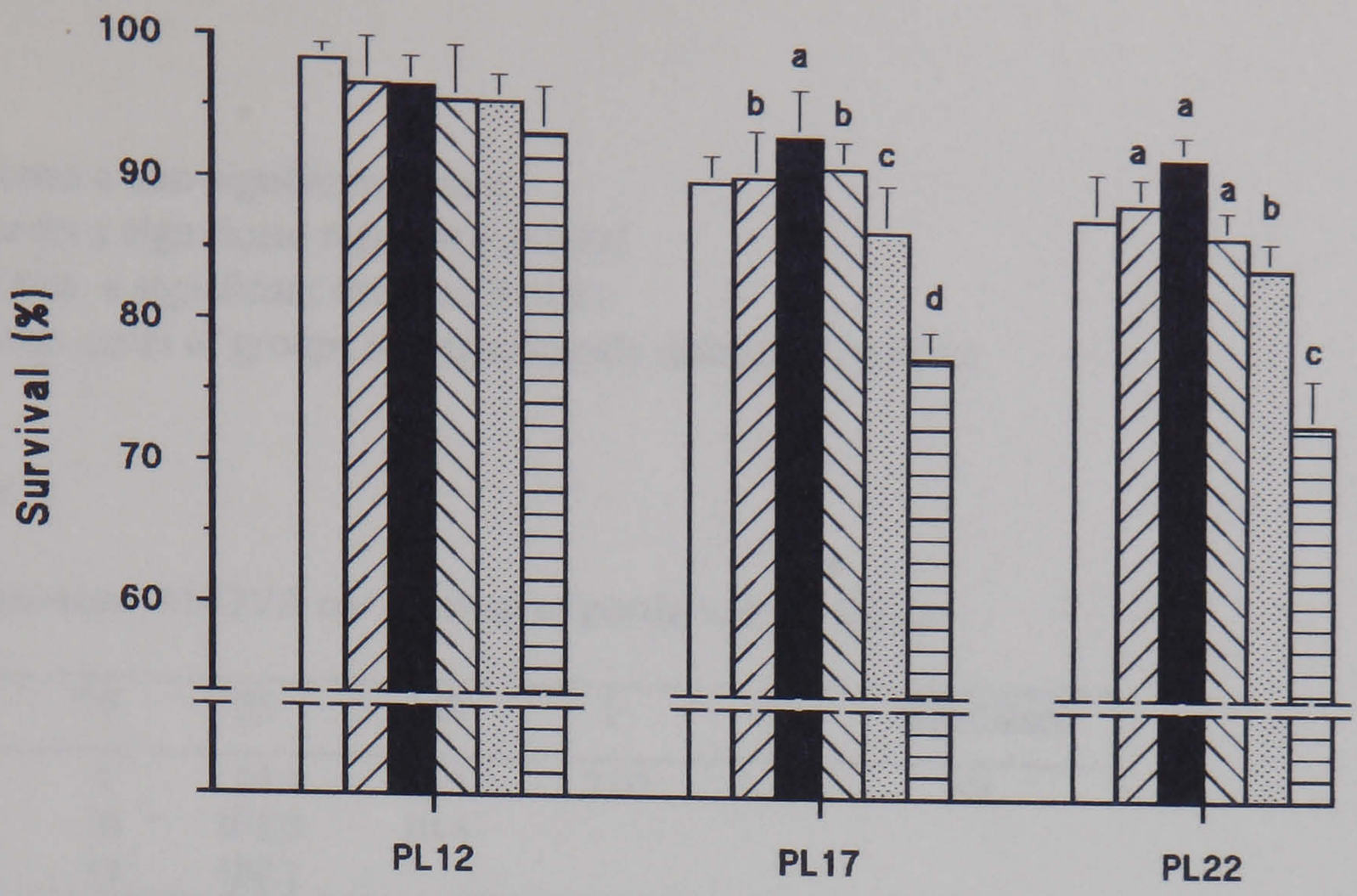
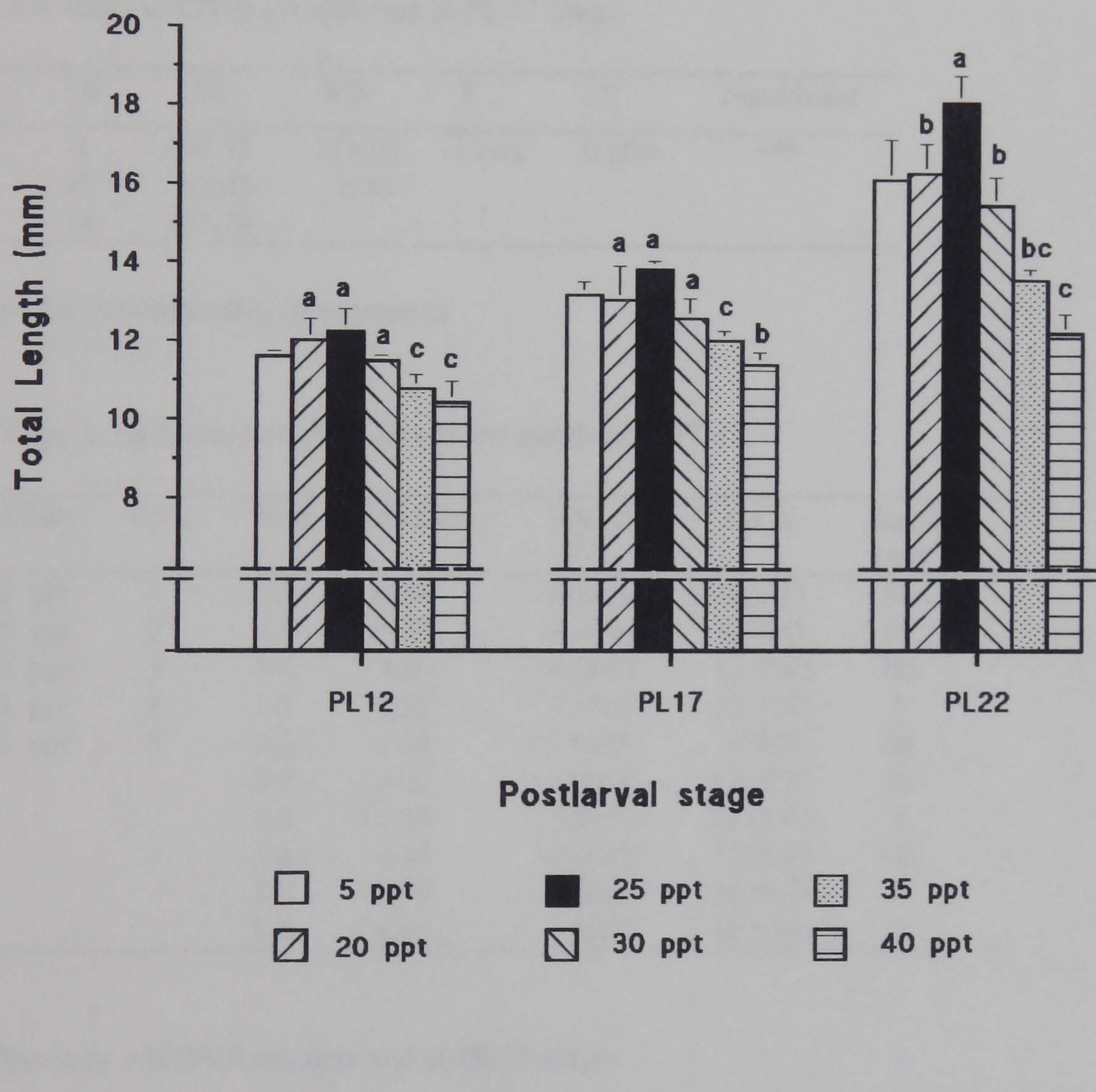


Figure 1b



Figures 1a, b. Survival (%) and total length (mm) of *P. indicus* postlarvae reared at salinities of between 5 and 40 ppt from PL7 to PL22. Each bar represents a mean ± s.d. (n=3). Treatments with the same superscripts are not significantly different (P>0.05).

(Ns) Indicates a non-significant result.
(***) Indicates a significant result at P≤ 0.001.
(**) Indicates a significant result at P≤0.01.
(*) Denotes pairs of groups are significantly different at p≤0.05.

Experiment 1

Table 1. One-way ANOVA on survival of postlarvae at PL12.

SOURCE	DF	SS	MS	F	P	Significant
Salinity	4	84.4	21.1	2.03	0.165	Ns
Error	10	103.8	10.4			
Total	14	188.2				

Bartlett's test for homogeneity of variances
F= 3.067, P= 0.15

Table 2a. One-way ANOVA on survival at PL17 stage.

SOURCE	DF	SS	MS	F	P	Significant
Salinity	4	447.33	111.08	13.64	0.000	***
Error	10	81.45	8.15			
Total	14	525.78				

Bartlett's test for homogeneity of variances
F= 1.462, P= 0.11

Table 2b. Tukey's pairwise comparison test on survival at PL17.

Mean	Salinity	Code	Test	diffmean	low_ci	up_ci	Signifi-cant
77.78	40 ppt	1	1-2	-3.00	-10.6643	4.6643	Ns
86.67	35 ppt	2	1-3	-0.78	-8.4443	6.8843	Ns
91.11	30 ppt	3	1-4	3.66	-4.0043	11.3243	Ns
93.33	25 ppt	4	1-5	12.55	4.8857	20.2143	*
90.33	20 ppt	5	2-3	2.22	-5.4443	9.8843	Ns
			2-4	6.66	-1.0043	14.3243	Ns
			2-5	15.55	7.8857	23.2143	*
			3-4	4.44	-3.2243	12.1043	Ns
			3-5	13.33	5.6657	20.9943	*
			3-6	8.89	1.2257	16.5543	*

Table 3a. One-way ANOVA on survival at PL22 stage.

SOURCE	DF	SS	MS	F	P	Significant
Salinity	4	618.28	154.57	34.78	0.000	***
Error	10	44.45	4.44			
Total	14	662.73				

Bartlett's test for homogeneity of variances
F= 3.143, P= 0.15

Table 3b. Tukey's pairwise comparison test on survival at PL22 stage.

Mean	Salinity	Code	Test	diffmean	low_ci	up_ci	Signifi-cant
73.33	40 ppt	1	1-2	-3.33	-8.987	2.327	Ns
84.44	35 ppt	2	1-3	2.22	-3.437	7.877	Ns
86.67	30 ppt	3	1-4	4.45	-1.207	10.107	Ns
92.22	25 ppt	4	1-5	15.56	9.903	21.217	*
88.89	20 ppt	5	2-3	5.55	-0.107	11.207	Ns
			2-4	7.78	2.123	13.437	*
			2-5	18.89	13.233	24.547	*
			3-4	2.23	-3.427	7.887	Ns
			3-5	13.34	7.683	18.997	*
			4-5	11.11	5.453	16.767	*

Table 4a. One-way ANOVA on total length at PL12.

SOURCE	DF	SS	MS	F	P	Significant
Stage	4	67.20	16.80	12.59	0.000	***
Error	142	189.40	1.33			
Total	146	256.89				

Bartlett's test for homogeneity of variances
F= 5.297, P= 0.12

Table 4b. Scheffé's pairwise comparison test for total length at PL12.

Mean	Salinity	Code	Test	diffmean	SE	low_ci	up_ci	Signifi-cant
10.44	40 ppt	1	1-2	-0.530	0.300326	-1.46736	0.40736	Ns
10.79	35 ppt	2	1-3	0.520	0.302860	-0.42527	1.46527	Ns
11.50	30 ppt	3	1-4	1.230	0.302860	0.28473	2.17527	*
12.55	25 ppt	4	1-5	1.580	0.300326	0.64264	2.51736	*
12.02	20 ppt	5	2-3	1.050	0.300326	0.11264	1.98736	*
			2-4	1.760	0.300326	0.82264	2.69736	*
			2-5	2.110	0.297770	1.18062	3.03939	*
			3-4	0.710	0.302860	-0.23527	1.65527	Ns
			3-5	1.060	0.300326	0.12264	1.99736	*
			3-6	0.350	0.300326	-0.58736	1.28736	Ns

Table 5a. One-way ANOVA on total length at PL17.

SOURCE	DF	SS	MS	F	P	Significant
Salinity	4	80.98	20.25	12.03	0.000	***
Error	99	166.62	1.68			
Total	103	247.60				

Bartlett's test for homogeneity of variances
F= 5.231, P= 0.12

Table 5b. Scheffé's pairwise comparison test for total length at PL17.

Mean	Salinity	Code	Test	diffmean	SE	low_ci	up_ci	Signifi-cant
11.45	40 ppt	1	1-2	-0.80	0.376737	-1.98264	0.38264	Ns
12.04	35 ppt	2	1-3	0.47	0.376737	-0.71264	1.65264	Ns
12.61	30 ppt	3	1-4	1.04	0.395922	-0.20286	2.28286	Ns
13.88	25 ppt	4	1-5	1.63	0.388927	0.40910	2.85090	*
13.08	20 ppt	5	2-3	1.27	0.409878	-0.01667	2.55667	Ns
			2-4	1.84	0.427579	0.49777	3.18223	*
			2-5	2.43	0.421110	1.10807	3.75193	*
			3-4	0.57	0.427579	-0.77223	1.91223	Ns
			3-5	1.16	0.421110	-0.16193	2.48193	Ns
			4-5	0.59	0.438357	-0.78607	1.96607	*

Table 6a. One-way ANOVA on total length at PL22.

SOURCE	DF	SS	MS	F	P	Significant
Salinity	4	374.33	93.58	25.86	0.000	***
Error	122	441.48	3.62			
Total	126	815.81				

Bartlett's test for homogeneity of variances
F= 2.808, P= 0.15

Table 6b. Scheffé's pairwise comparison test for total length at PL22.

Mean	Salinity	Code	Test	diffmean	SE	low_ci	up_ci	Signifi-cant
12.26	40 ppt	1	1-2	-1.37	0.495474	-2.91981	0.17981	Ns
13.63	35 ppt	2	1-3	-3.28	0.491530	-4.81747	-1.74253	*
15.54	30 ppt	3	1-4	-5.94	0.504097	-7.51678	-4.36322	*
18.20	25 ppt	4	1-5	-4.10	0.504097	-5.67678	-2.52322	*
16:36	20 ppt	5	2-3	-1.91	0.487279	-3.43417	-0.38583	*
			2-4	-4.57	0.499952	-6.13381	-3.00619	*
			2-5	-2.73	0.499952	-4.29381	-1.16619	*
			3-4	-2.66	0.496044	-4.21159	-1.10841	*
			3-5	-0.82	0.496044	-2.37159	0.73159	Ns
			4-5	1.84	0.508499	0.24945	3.43055	*

Table 7. Survival (%) and total length (mm) (data for Figures 1a, b) of *P. indicus* postlarvae reared at different salinities from PL7 to PL22. Each value is a mean ± s.d. (n=3).

Survival (%)						
Stages	5 ppt	20 ppt	25 ppt	30 ppt	35 ppt	40 ppt
PL7	100.00	100.00	100.00	100.00	100.00	100.00
PL12	98.33±1.12	96.67±3.34	96.67±1.92	95.56 ±3.85	95.55±1.92	93.33±3.33
PL17	89.99±1.92	90.33±3.34	93.33±3.34	91.11±1.92	86.67±3.33	77.78±1.92
PL22	87.77±3.34	88.89±1.92	92.22±1.62	86.67±1.00	84.44±1.93	73.33±3.33
Total length (mm)						
PL7	9.15±0.07	9.15±0.07	9.15±0.07	9.15±0.07	9.15±0.07	9.15±0.07
PL12	11.60±0.14	12.02±0.54	12.55±0.56	11.50±0.14	10.79±0.36	10.44±0.54
PL17	13.21±0.33	13.08±0.88	13.88±0.19	12.61±0.50	12.04±0.26	11.45±0.33
PL22	16.19±1.04	16.36±0.76	18.20±0.67	15.54±0.73	13.63±0.27	12.26±0.49

Experiment 2

Figures 2a and 2b show that *P. indicus* postlarvae sustain higher survival and better growth (TL) at lower salinities than at higher salinities between PL20 to PL60 stages. Although 10 ppt salinity sustained a very high survival and growth rate from PL20 to PL45, survival rate of the animals started to decline sharply after stage PL45 indicating that this low salinity becomes stressful to late postlarvae (Figure 2a). Final survival of animals subjected to this salinity was only 13 % at PL60 stage (Tables 8 and 11). Survival obtained from this treatment did not show a linear relation and hence it was excluded in the statistical calculations.

Table 8 summarises growth rate (mm day^{-1} TL), mortality rate (% day^{-1}) between PL20 and PL60, total length (mm), survival (%), body wet-weight (g) and total biomass (g) and increase in body weight per mm increase in TL at PL60. Survival and growth rate at 20 ppt salinity were significantly ($P<0.01$) better than at other salinities tested in this trial (Tables 9 and 10). Growth and survival rates at higher salinities, particularly 50 ppt S, were significantly inferior to those recorded at lower salinities ($P<0.01$). Optimum culture salinity for the postlarvae (PL20-PL60) appears to be between 20 and 30 ppt at 29-31 °C. While postlarvae cultured at 20 ppt S reached 37 mm in total length with 72 % survival within 40 days of culture, animals at 50 ppt salinity were only 27 mm with a 52 % survival (Table 8). PLs cultured at 40 ppt also exhibited slow growth and a high mortality rate by PL60. Tables 9-10 show no significant differences ($P>0.05$) between the growth and survival rate of postlarvae cultured at 30, 35 ppt S.

Results of individual wet-weight, total biomass and increase in body weight per mm total length (TL) also confirm the above findings. Figure 3 shows that, at stage PL60, both total biomass and individual weight of the specimens cultured at lower salinities were significantly greater ($P<0.01$) than those at higher salinities. Table 8 displays that 20 ppt salinity gave the high-

est biomass (3.70 g), mean individual wet-weight (0.28 g) and weight per mm total length (7.54 mg^{-1} mm^{-1}) than at all other salinities, whereas 50 ppt resulted in the lowest performance (1.39 g and 0.13 g and 4.84 mg^{-1} mm^{-1} respectively).

Figure 4 plots survival and body wet weight per mm TL obtained by Bukhari et al., (1994), who used identical experimental procedures, together with the present trial for postlarvae reared at different salinities from PL20 to PL60. From this figure, it is clear that the Red Sea *P. indicus* postlarvae survive and grow better in high saline conditions (50 ppt S), whereas Indian *P. indicus* prefer lower salinities. The best survival, growth and yield (biomass) for Red Sea *P. indicus* were reported to be from animals previously acclimatised for 10 days and reared at 50 ppt by these authors. Our Indian *P. indicus* strain showed highest body weight per mm TL at low salinities (20 ppt) and lowest at high salinities (50 ppt). However, with the Red Sea *P. indicus*, Bukhari et al., (1994) found the highest body weight per mm TL at 50 ppt (4.6 mg^{-1} mm^{-1}) and lowest (0.72 and 2.15 mg^{-1} mm^{-1}) at 15 ppt and 25 ppt salinities respectively (see Figure 4). In the present study, optimal salinity appears to fall between 20 and 30 ppt S for the Indian strain of *P. indicus*.

Figure 2a

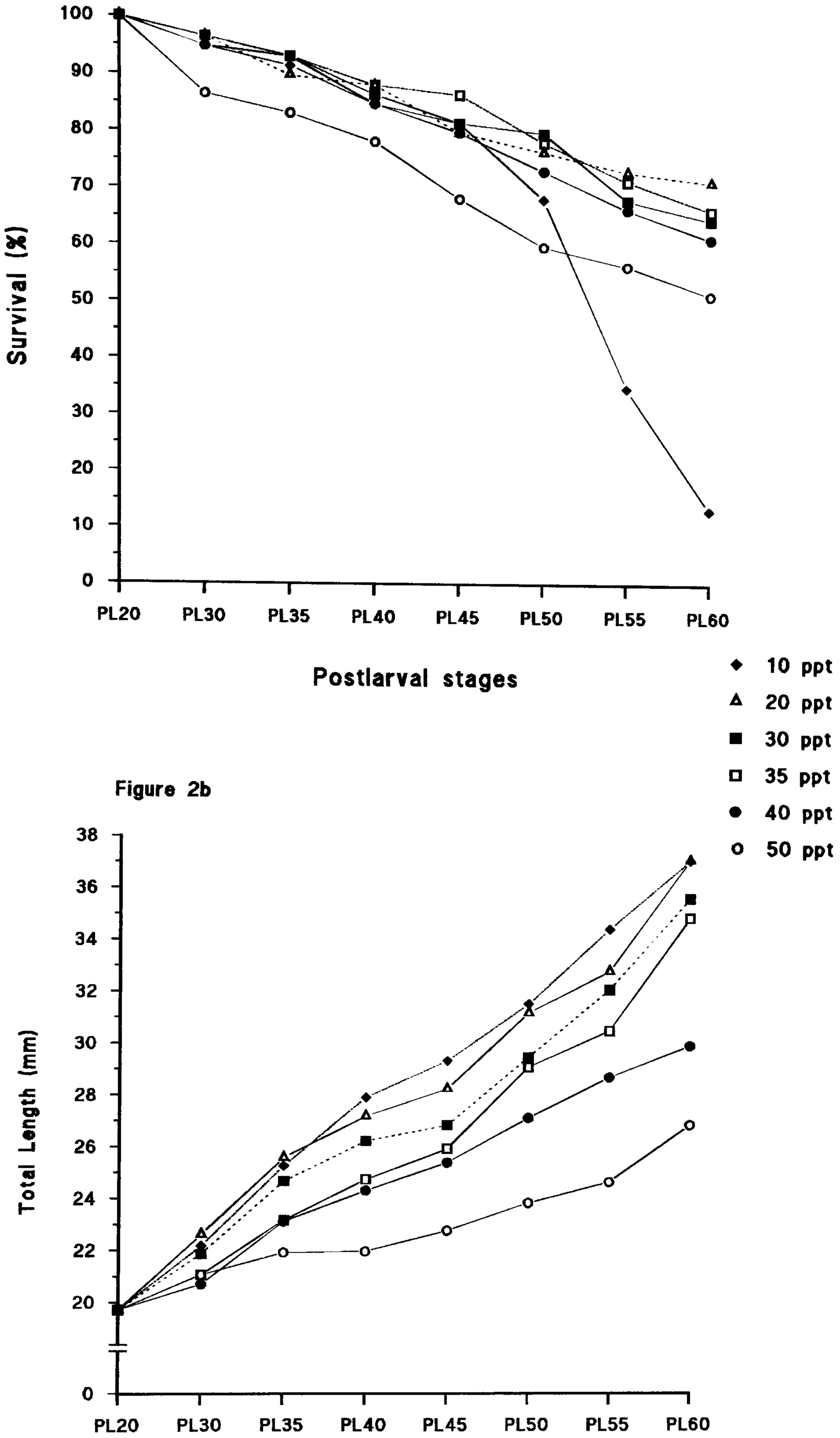


Figure 2b

Figures 2a, b. Survival (%) and total length (mm) of *P. indicus* postlarvae reared at salinities of between 10 and 50 ppt from PL20 to PL60. Each symbol is a mean (n=3).

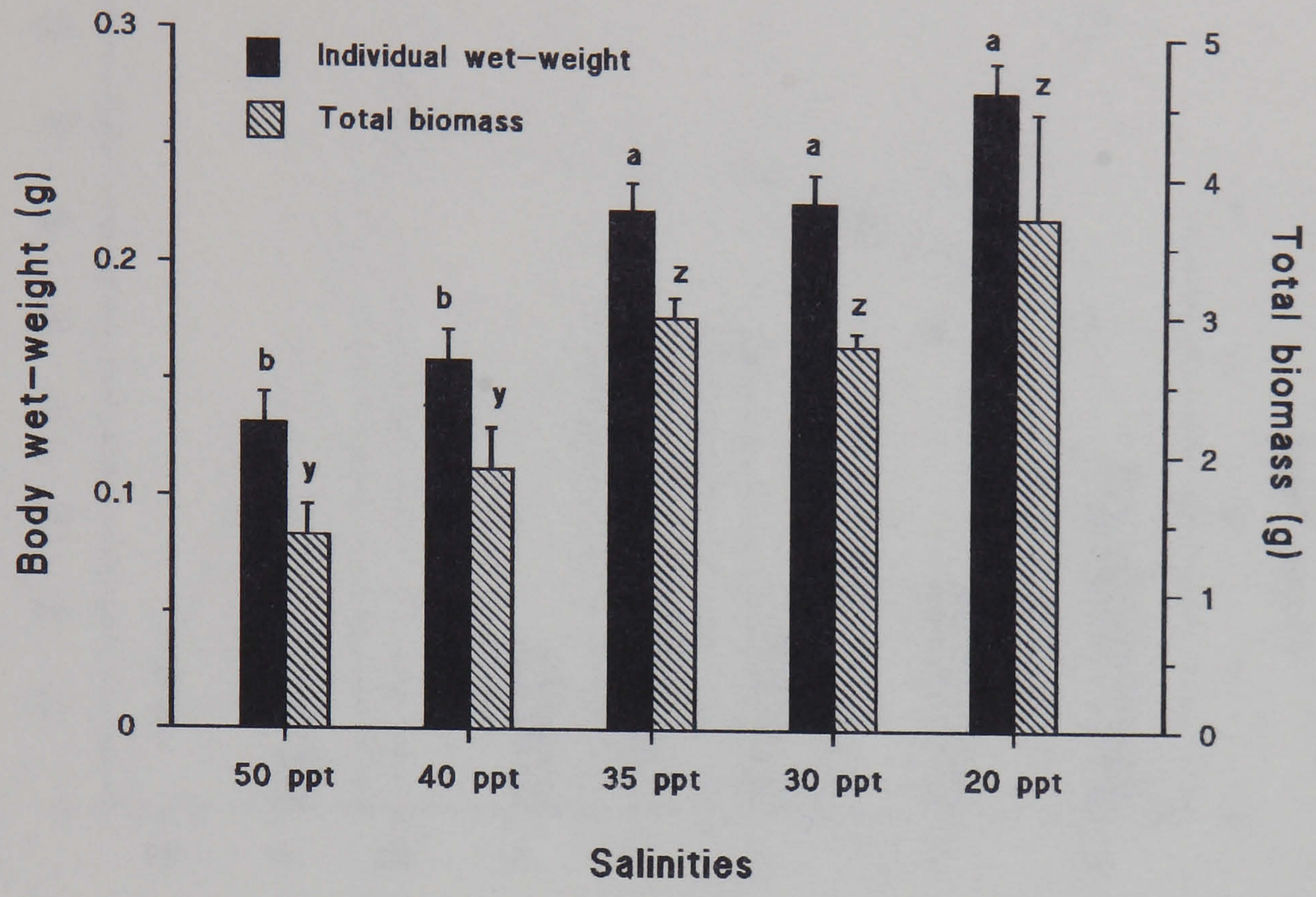


Figure 3. Body–wet weight and total biomass of *P. indicus* postlarvae at stage PL60 reared at different salinities from PL20 to PL60. Each bar represents the mean ± s.d. from three replicates for each test salinity. Treatments with the same superscripts are not significantly different (P>0.05).

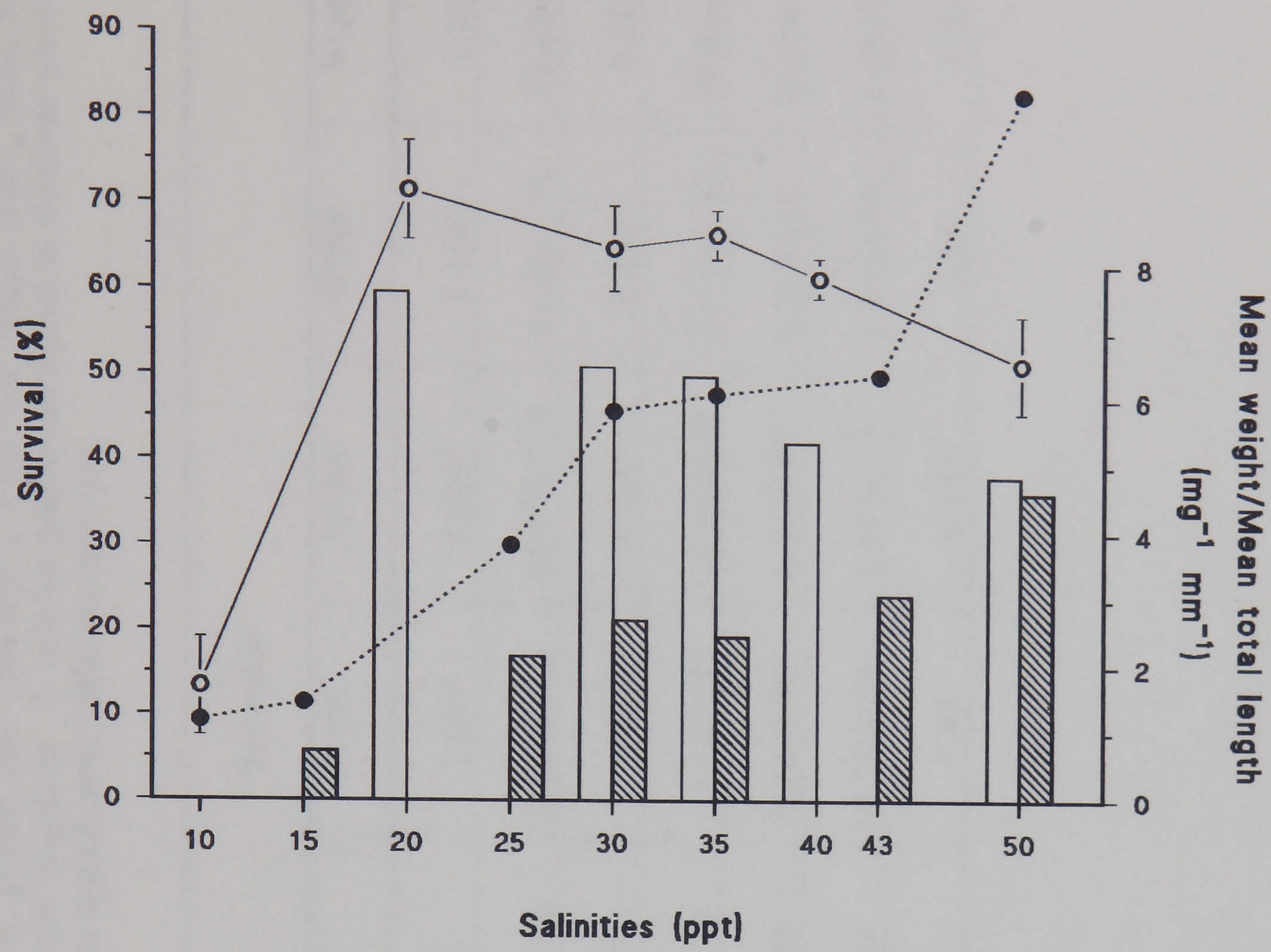


Figure 4. Survival (%) and increase in body wet–weight per mm total length (mg^{-1} mm^{-1}) in *P. indicus* postlarvae from the Red Sea (Bukhari et al.,1994) and India (present study).

Table 8. Mortality rate (% day^{-1}), final survival (%), growth rate (mm day^{-1}), final total length (mm), mean weight (g), total biomass (g) and increase in weight per mm total length in *P. indicus* postlarvae reared in different salinities from PL20 to PL60. Values with same superscripts are not significantly different ($P<0.05$).

	Salinities					
	10 ppt	20 ppt	30 ppt	35 ppt	40 ppt	50 ppt
Mortality rate (% day^{-1})	-	0.857^{a}	1.083^{b}	1.024^{b}	1.168^{c}	1.262^{d}
Final survival (%)	13.33±5.78	71.68±5.78	65.00±5.00	66.67±2.89	61.67±2.36	51.67±5.77
Growth rate (% day^{-1})	0.476^{a}	0.442^{a}	0.424^{b}	0.434^{b}	0.299^{c}	0.183^{d}
Final total length (mm)	37.13±1.16	37.13±1.82	35.65±0.56	34.86±0.89	29.93±1.95	26.85±0.94
Mean weight (g)	-	0.28±0.01	0.23±0.01	0.22±0.01	0.16±0.01	0.13±0.02
Total biomass (g)	-	3.70±0.77	2.77±0.10	2.97±1.14	1.87±0.30	1.39±0.22
Mean weight/ final total length (mg/mm)	-	7.54	6.45	6.311	5.35	4.84

(Ns) Indicates a non-significant result.
(***) Indicates a significant result at P≤ 0.001.
(**) Indicates a significant result at P≤0.01.
(*) Denotes pairs of groups are significantly different at p≤0.05.

Experiment 2

Table 9a. Two-way ANOVA with days as a covariate on survival of *P. indicus* postlarvae from PL20 to PL60.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Salinity	5	860.88	11.46	2.29	0.19	0.962	
Days	1	1002.39	1002.39	1002.39	83.59	0.000	
Repl(Salinity)	12	88.72	78.69	6.56	0.55	0.856	
Salinity*days	5	206.14	206.14	41.23	3.44	0.024	*
Repl*Days(salinity)	12	85.38	85.38	7.12	0.59	0.820	
Error	18	215.85	215.85	0.59			
Total	53	2459.36					

Table 9b. Comparison of the mortality rates of *P. indicus* postlarvae reared at different salinities (20-50 ppt S) from PL20 to PL60.

	Mortality rate (% day^{-1})	Slopes	Coeff.	Stdev.	t-value	P	Significant
Constant			100.762	0.9700	103.87	0.000	
Weeks			-5.4048	0.2169	-24.92	0.000	
Weeks*Salinity							
50 ppt	1.262	-6.310	-0.9048	0.4338	-2.09	0.040	*
40 ppt	1.168	-5.839	-0.4881	0.4338	-3.13	0.264	Ns
35 ppt	1.024	-5.119	0.2857	0.4338	0.66	0.512	Ns
30 ppt	1.083	-5.417	-0.0119	0.4338	-0.03	0.978	Ns
20 ppt	0.857	-4.286	1.1190	0.4338	2.58	0.012	*

Table 10a. Two-way ANOVA with days as a covariate on growth of *P. indicus* postlarvae from PL20 to PL60 .

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Salinity	5	484.609	2.201	0.440	0.19	0.967	
Days	1	1070.739	1023.319	1023.319	437.18	0.000	
Repl(Salinity)	12	29.913	20.638	1.720	0.73	0.717	
Salinity*days	5	96.921	93.380	18.676	7.98	0.000	***
Repl*Days(salinity)	12	18.695	18.695	1.558	0.67	0.785	
Error	399	933.952	933.952	2.341			
Total	434	2634.831					

Table 10b. Comparison of the growth rate of *P. indicus* postlarvae reared at different salinities (20-50 ppt S) from PL20 to PL60.

	Growth rate (mm day^{-1})	Slopes	Coeff.	Stdev.	t-value	P	Significant
Constant			19.6206	0.1821	107.77	0.000	
Weeks			1.8813	0.0418	44.94	0.000	
Weeks*Salinity							
50 ppt	0.183	0.917	-0.9639	0.0905	-10.65	0.000	***
40 ppt	0.299	1.496	-0.3856	0.0906	-4.25	0.000	***
35 ppt	0.434	2.171	0.2899	0.0911	3.18	0.002	***
30 ppt	0.424	2.122	0.2402	0.0925	2.60	0.010	**
20 ppt	0.442	2.209	0.3276	0.0918	3.57	0.000	***
10 ppt	0.476	2.379	0.4919	0.1044	4.71	0.000	***

Table 11a. Survival (%) data of *P. indicus* (in Figure 2a) postlarvae reared at different salinities from PL20 to PL60. Each value is a mean ± s.d. (n=3).

	Survival (%)					
Stages	10 ppt	20 ppt	30 ppt	35 ppt	40 ppt	50 ppt
PL20	100.00	100.00	100.00	100.00	100.00	100.00
PL30	95.00±5.00	96.67±2.89	96.67±5.77	96.67±2.89	95.00±5.00	86.67± 2.89
PL35	91.67±5.77	90.00±5.77	93.33±2.89	93.33±2.89	93.33±2.89	83.33± 2.89
PL40	85.00±5.00	88.33±5.00	86.67±2.89	88.33±2.89	85.00±0.00	78.33± 2.89
PL45	81.67±2.89	80.00±2.89	81.67±2.89	86.67±2.89	80.00±2.89	68.33± 2.89
PL50	68.33±2.89	76.67±2.89	80.00±5.77	78.33±5.77	73.33±2.89	60.00±13.23
PL55	35.00±10.00	73.33±2.89	68.33±2.89	71.67±7.64	66.67±7.64	56.67±10.41
PL60	13.33±5.78	71.68±5.78	65.00±5.00	66.67±2.89	61.67±2.36	51.67± 5.77

Table 11b. Total length (mm) data of *P. indicus* (in Figure 2b) postlarvae reared at different salinities from PL20 to PL60. Each value is a mean ± s.d. (n=3).

	Total length (mm)					
Stages	10 ppt	20 ppt	30 ppt	35 ppt	40 ppt	50 ppt
PL20	19.71±0.33	19.71±0.33	19.71±0.33	19.71±0.33	19.71±0.33	19.71±0.33
PL30	22.17±0.75	22.61±0.48	21.87±0.42	21.06±0.36	20.69±0.29	21.06±1.14
PL35	25.27±0.31	25.59±0.20	24.68±0.52	23.17±0.29	23.11±0.86	21.92±0.23
PL40	27.91±0.54	27.19±0.42	26.22±0.45	24.74±0.43	24.31±0.53	21.96±0.37
PL45	29.33±0.71	27.24±0.80	26.84±1.18	25.93±0.53	25.40±0.35	22.76±0.28
PL50	31.54±2.46	31.20±1.06	29.48±1.03	29.10±0.89	27.12±1.26	23.83±0.76
PL55	34.46±1.32	32.82±0.97	32.11±0.94	30.51±0.63	28.71±1.26	24.64±1.17
PL60	37.13±1.16	37.13±1.82	35.65±0.56	34.86±0.89	29.93±1.95	26.85±0.94

DISCUSSION

Present results indicate that *P. indicus* is a very successful hypo and hyper osmoregulator during early postlarval stages. It can withstand an abrupt salinity change from 30 ppt S to 20 ppt and 40 ppt S without adverse survival. However, in the first experiment, 100 % mortality was recorded in 24 h when the animals were transferred without acclimation from a salinity 30 ppt to 5 ppt S water. Sudden transfer to 10 ppt salinity also caused 80 % mortality, indicating that early postlarvae of this species cannot tolerate salinity changes of greater than 10 ppt S day^{-1}. Bukhari (1994) also obtained only 0.04% survival when he transferred *P. indicus* postlarvae (PL10) originating from the Red Sea directly from 30 ppt to 10 ppt S and cultured them until PL20 stage. As postlarvae were successfully acclimatised to 5 ppt S over 48 h, it is recommended that an acclimation period of this length is necessary if postlarvae are to be stocked into brackish nursery ponds of 5 to 10 ppt salinity. This confirms findings of Parado-Estepa et al., (1987) who report that *P. indicus* at 5-10 g are also able to osmoregulate in salinities of between 8 and 40 ppt, but need an acclimation time of 0.5-2.0 days. Results obtained from the current study at 5 ppt are similar to those of Raj and Raj (1982) who report 5 ppt S as one of the best salinity levels during their experiments with *P. indicus* wild postlarvae, previously acclimatised for 8 days.

Like many other penaeids, *P. indicus* is a euryhaline species growing in a wide range of salinities. Present results indicate that, although the PLs of this species can tolerate high saline conditions, they clearly grow and survive better in low salinities (Figures 1-2). Between PL7 and PL22, animals show consistently better survival and growth at 20 and 25 ppt S than at high salinities (40 ppt S) indicating that their optimal salinity is in the lower range (Table 7). This agrees with Raj and Raj (1982) who studied *P. indicus* postlarvae and juveniles captured from the wild (no PL stage indicated) and

demonstrated that postlarvae prefer lower salinities. Based on present results and those of Raj and Raj (1982), 25 ppt salinity is the best recommended salinity for Indian *P. indicus* strains during nursery postlarval rearing at 29-31 °C. In Chapter 1, it was found that 25 ppt S is optimal for the larval culture of *P. indicus* from PZ1 to PL1. Staples and Heales (1991) also obtained the greatest biomass increase in *P. merguiensis* at 25 ppt S and 28 °C. Dall (1981) interprets this low salinity preference shown by the postlarvae of penaeids as a useful adaptation to their natural nursery habitats.

Present postlarvae cultured at 10 ppt S showed poor survival in this medium after PL40 stage suggesting that animals at PL35-40 become more stenohaline preferring higher salinities. Postlarvae and juveniles are more capable of withstanding lower salinities than adults (Charmantier, 1987) and tolerance to euryhalinity may be lost gradually with development under stable conditions (Dall, 1981). Similarly, Staples (1980) also notes that 10 ppt S is a critical salinity level for large *P. merguiensis*. It appears from present results that *P. indicus* is not a suitable candidate for culture at salinities lower than 10 ppt, particularly from PL45 onwards. This is confirmed by Primavera (1984; cited in Parado-Estepa et al., 1987), who observed mass mortality of this species during culture in grow-out ponds at salinities lower than 10 ppt, and Parado-Estepa et al., (1987) who report that this species is incapable of osmoregulating efficiently below 8 ppt S. Therefore, in agreement with Bukhari et al., (1994), it is suggested that PL35-40 stage is probably the ideal time to transfer *P. indicus* postlarvae to on-growing ponds.

Food consumption and conversion ratio are correlated with temperature and salinity of culture water. In the present study, it was observed that food consumption of animals reared at high salinities was much lower in comparison to those cultured at low salinities. Since the temperature was constant during the experiments, variation in food consumption was mainly related to

salinity. As a result of high food consumption, low salinities consistently gave superior growth and survival as occurs in the natural estuarine nursery habitat of the postlarvae (Venkataramaiah et al., 1972). Hence, after larval rearing, animals can be placed directly without acclimatisation in nursery water at 20-25 ppt S where salinity fluctuations do not exceed 10-15 ppt S per day.

Whilst optimal salinity for larval culture (20-25 ppt S) did not differ significantly between the present Indian race and Red Sea race of *P. indicus*, Figure 4 demonstrates that postlarval salinity preferences are very different. Significant differences in the growth rate at 50 ppt S become apparent at PL40 with Indian stock showing slower growth than at other salinities (Figure 2), whereas Red Sea stock shows the fastest growth at this salinity level (Bukhari et al., 1994). By PL60, Indian stock shows significantly slower growth at 40 and 50 ppt S, whilst Red Sea stock shows significantly faster growth at 43 and 50 ppt S. The Red Sea strain attained the highest yield at 50 ppt S whereas our Indian strain gave the maximum yield at 20 ppt S. Final body wet weight (at PL60) per mm TL between the two shrimp populations also differed at extreme salinities. While the Red Sea *P. indicus* showed an increase in body weight (0.72 to 4.8 mg^{-1} mm^{-1} TL) with increasing salinity from 15 to 50 ppt, the present Indian race exhibits a decline in body weight (7.54 to 4.85 mg^{-1} mm^{-1}) TL with an increase in salinity from 20 to 50 ppt (Figure 4).

The salinity preferences of Indian *P. indicus* postlarvae cultured in the present study follow a similar pattern to wild Indian postlarvae (Raj and Raj, 1982), with slower growth and higher mortality at higher salinities, despite culture through several generations in Tahiti and Europe at 35 ppt S. In contrast, Red Sea *P. indicus* appear to be a distinct physiological strain adapted to high saline conditions. This has important repercussions for the aquaculture industry as it becomes possible to select broodstock physiolo-

gically suited to particular nursery and grow-out salinity regimes. Harpaz and Karplus (1991) suggest that the difference in salinity tolerance between *P. semisulcatus* populations in the Philippines (Valencia, 1977) and in the Mediterranean (Samocha, 1980; cited in Harpaz and Karplus, 1991) may be due to inherent differences between the two populations. The difference in salinity tolerance between two separate populations of Australian penaeids was ascribed to localised inherited abilities which are dependent on salinity-temperature history of the parent stock (Preston, 1985a). In the present study, whilst optimal salinity for larval culture (20-25 ppt S) did not differ significantly between the two populations, postlarval salinity preferences are very different (Figure 4). Therefore, in agreement with Harpaz and Karplus (1991), the present results suggest that inherent differences may exist between the Red Sea and our Indian *P. indicus* populations. It remains to be seen whether Red Sea *P. indicus,* now about to be cultured through a second generation in Europe in sea water of 35 ppt S, will retain tolerance to a high saline environment.

CHAPTER 3

NEMATODES AS AN ALTERNATIVE LIVE FEED FOR CARIDEAN AND PENAEID LARVAE.

In this chapter *P. indicus* = *Penaeus indicus*, *P. elegans* = *Palaemon elegans*, *P. redivivus* = *Panagrellus redivivus*.

Parts of this chapter were presented in 'WORLD AQUACULTURE' 94' New Orleans, USA, January 14-18, 1994.

Title: Growth and survival of *Penaeus indicus* larvae fed on the nematode *Panagrellus redivivus*.

Authors: Fletcher, D. J., Fisher, C. M., Kumlu, M. and Rodgers, P. B.

Published: World Aquaculture Society, Book of Abstracts, p. 109.

INTRODUCTION

The major objective in penaeid aquaculture is to establish adequate feeding regimes that promote reasonably 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; Watanabe et al., 1983; Jones, 1988; Léger and Sorgeloos, 1992; Jones et al., 1993). Although *Artemia* is a very suitable feed 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), its high price (Langdon et al., 1985), availability, nutritional quality and variations in hatching efficiency between different strains (Watanabe et al., 1983; Léger et al., 1986) are also well known drawbacks. In addition, *Artemia* consume algal feeds and grow too 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 PZ1 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 larvae 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*, *Caenorhabditis elegans*, *Steinernema feltiae*) for the culture of two commercially important shrimp species; a marine penaeid, *Penaeus indicus* and a freshwater prawn, *Macrobrachium rosenbergii*. In order to obtain some preliminary experience in the use of nematodes as larval feeds, initial trials were performed with the larvae of a native prawn, *Palaemon elegans*. After a few experiments with caridean larvae, most of the research was concentrated on the penaeid larvae of *P. indicus* since *P. elegans* and *M. rosenbergii* larvae were unable to survive on the nematodes. Following preliminary trials, 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 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

Different species of live nematodes were fed to larvae of two caridean species, *P. elegans* and *M. rosenbergii* as substitutes for live *Artemia* in their culture. Conventional live feeds for *P. indicus* larvae (algae during protozoeal stages and *Artemia salina* nauplii during mysis and postlarval stages) were also replaced using the nematode *P. redivivus.*

Rearing procedures

Basic experimental procedures were identical for all species except where otherwise stated. The larval trials were performed in 2-l round bottom glass flasks incubated in a water bath at the appropriate temperature for each species (as described later). Sea water was filtered (0.2 µm) and irradiated with ultra-violet light (UV) to prevent extraneous food sources and to reduce bacterial contamination. Aeration sufficient to maintain continuous prey/predator interaction without damaging the prawn larvae was obtained via silicone rubber tubing and a single glass pipette at the tip supplied with compressed air. Complete water exchanges were performed every day or every other day when the larvae were also counted and a sample of 10-14 larvae were staged and measured from the tip of the rostrum to the end of telson (Total length = TL) for each replicate. Except where otherwise stated, 50% of the culture water of each individual flask was changed every day when algal and prey counts were estimated and an appropriate amount of fresh food was added to maintain specific feeding regimes for each species. *Artemia* cysts (INVE AQUACULTURE, Belgium) were continuously hatched in 2-l glass flasks at 26-28 °C at 32 ppt S during the experiment. Newly hatched nauplii were separated from the shells of the cysts and counted five times to estimate their density.

Nematode cultures

Live nematodes were obtained from Agricultural Genetics Company (AGC), West Sussex, UK soaked in small cubic sponges (10 million in 3 g sponge in

10 ml of water) and kept at 4 °C in an ordinary refrigerator. They were released in distilled water, concentrated and counted (three times) in a rafter cell counting chamber before they were fed to the larvae. Size range of the nematodes were generally between 150 μ and 900 μ. Nematodes were produced in liquid medium axenically or 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*

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

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

Culture temperature: 22 °C.

Shaking incubator : 170 rpm

An inoculum of 2000 nematodes ml^{-1} was added to the flasks which had been previously (24 h) inoculated with *Escherichia coli*. The flasks were incubated at 22 °C for 10-22 days in an orbital incubator. Nematodes were harvested when maximum populations occurred, at approximately 150,000 nematodes ml^{-1}. The nematodes were extracted from the media by centrifugation and cleaned to remove any residual medium by a repetitive suspension, sedimentation and decantation in fresh water. They were packed in 15x18 cm high density polyethylene bags containing 2 g 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-l 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 (Fisher, pers. comm.). Generally, nematodes were regularly obtained from AGC and used in 1-3 days of receipt from the company during the experiments.

Caridean larvae

A feeding experiment was performed to test whether the nematode, *Caenorhabditis elegans* can be used as food for *P. elegans* larvae. Two dif-

ferent trials were conducted in an attempt to replace *Artemia* with four different nematode species, *C. elegans, Steinernema carpocapsae*, *Steinernema feltiae* and *P. redivivus* during the culture of *M. rosenbergii* larvae from Z1 stage onwards.

Palaemon elegans

Ovigerous females were obtained from the Menai Strait and kept in 50-l glass aquaria at 25 °C until the larvae were released according to the lunar cycle. Newly hatched larvae (Z1) were stocked at a density of 50 l^{-1} in filtered (0.2 µm) and UV-treated sea water in 2-l flasks and temperature during experiment was maintained at 25 °C in a thermostatically controlled water bath. The nematode, *C. elegans,*(450-500 µm in length) was fed to the larvae at a concentration 30 ml^{-1}. The control larvae were either fed newly hatched *Artemia* nauplii at 10 ml^{-1} or starved. 50 % of the rearing water in 2-l flasks was replaced every day. Survival and growth of the larvae were assessed every other day from two replicates. The experiment was terminated when all the larvae on the nematode diet had died on day 9.

Macrobrachium rosenbergii

Two experiments were conducted with *M. rosenbergii* larvae to assess whether they could be fed on different species of nematodes. Larvae were obtained from individual female broodstock held in a 100-l tank at 29 °C in freshwater. Newly hatched Z1 stage larvae were stocked at a density of 50 larvae l^{-1} in water at 29 °C and 12 ppt S in 2-l experimental flasks. Temperature of the rearing water was maintained at 29 °C ± 0.5 by keeping the flasks in a thermostatically controlled water bath. The brackish water was obtained by mixing distilled water with filtered and UV-treated sea water.

Experiment 1

In the present experiment, two species of nematode, *C. elegans* and *S. carpocapsae* were initially fed at two different densities as described in Table 1.

On day 4, since none of the larvae had full guts with the nematodes, it was decided to feed the larvae on the larger nematode species *S. feltiae*. Nematode concentrations were also increased as in Table 1 to ensure that sufficient prey was available to the larvae at all times. All these nematode species were cultured on a medium enriched with 25 % capelin oil to improve their fatty acid profile for the prawn larvae. 50 % of the culture water was exchanged every day when new nematode feeds were added to the culture. Growth and survival was assessed every other day from two replicates.

Table 1. Feeding levels of *Artemia* and three different species of nematodes (ml^{-1}) used in the present experiment to feed *M. rosenbergii* larvae from Z1 stage.

Days	*Artemia* (ml^{-1})	*C. elegans* (ml^{-1})		*S. carpocapsae* (ml^{-1})		Starved
0	10	75	150	75	150	-
2	10	75	150	75	150	-
		S. feltiae (ml^{-1})				
4	15	150	225	125	175	-
6	15	150	225	125	175	-

Experiment 2

Experimental procedures were identical to those used in Experiment 1. Larvae (Z1 stage) of *M. rosenbergii* were stocked in 2-l flasks and fed with the nematode *P. redivivus* at a concentration of 10, 20 and 30 nematodes ml^{-1} on days 0, 2 and 4 respectively. Control larvae were fed newly hatched *Artemia* at a density of 10 nauplii ml^{-1}. In another treatment larvae were starved as another control. Also, larvae were observed in petri dishes under a binocular microscope to determine whether they could ingest and digest the nematodes.

Penaeid larvae

For each experiment, *P. indicus* larvae were obtained from individual females held in 50-l spawning tank at 28 °C and 33 ppt salinity. After the spawning, 50 % of the sea water of the tank was changed with filtered (0.2 µm) and UV-treated sea water. Protozoea 1 (PZ1) stage larvae were treated

with an antibiotic (furazolidone) at 0.2 ppm before the larvae were stocked into experimental flasks at a density of 100 larvae l^{-1} and fed the experimental diets. Experiments were carried out in a thermostatically controlled water bath at 28 ± 0.5 °C. Continuous aeration was supplied via silicone rubber tubing with glass tubes at the tip to ensure sufficient amount of oxygen and prey/predator movement in the rearing water. Except in the first experiment, salinity was maintained at 25 ppt (optimal to rear *P. indicus* larvae - See Chapter 1), throughout all experiments by mixing filtered (0.2 µm) and UV-irradiated sea water with distilled water.

Experiment 1

P. indicus larvae (PZ1 stage) fed 50 cells μl^{-1} *Tetraselmis chuii* and *Rhinomonas reticulata* (1:1) plus Frippak microencapsulated diets (INVE AQUACULTURE, Belgium) until the experiment started at PZ2/3 stages. The larvae were stocked at a density of 30 larvae l^{-1} in 2-l experimental flasks to assess survival and growth of mysis larvae on the nematode *P. redivivus* or *Artemia* nauplii.

Table 2. Feeding regimes used to rear *P. indicus* larvae from stage 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

Larval feeding regimes are shown in Table 2. Algal feeds were fed to the larvae along with *P. redivivus* and *Artemia* until day 3 when the algal cell concentration was halved. On day 4, the algal feed totally ceased and *P. indicus* larvae were fed on either *Artemia* or the nematode diet until the end

of the experiment. Growth and survival data was analysed using one-way ANOVA in Minitab following Bartlett's test for homogeneity of variances.

Experiment 2

Two nematode species, *P. redivivus* and *C. elegans* were fed to PZ1 stage of *P. indicus* larvae at a density of 25 nematodes ml^{-1} in an attempt to see whether the larvae would survive on these two nematode species from the first feeding stage (PZ1). Larval growth and survival measurements were performed every other day from three replicates for each treatment. These nematode species were obtained from the AGC continuously during the experiment. Control larvae were reared on mixed live algae of *T. chuii* (25 cells μl^{-1}) and *S. costatum* (35 cells μl^{-1}) until PZ3/M1 stage when the experiment had to be terminated due to heavy mortalities observed with the larvae fed the nematodes.

Experiment 3

It was thought that the previous experiment collapsed because possibly the larvae were not fed at appropriate feeding level of nematodes. Thus, in this experiment the nematode *P. redivivus* was fed to the larvae at four different concentrations (30, 40, 50 and 60 ml^{-1}) from stage PZ1 to assess adequate nematode feeding density in the absence of microalgae. Larvae were stocked in 2-l flasks at a density of 100 larvae l^{-1} at PZ1 stage. The control larvae were fed live mixed algae *T. chuii* (25 cells μl^{-1}) /*S. costatum* (35 cells μl^{-1}). Experiments were conducted in triplicates for each treatment. Larval count and total length measurement were performed every other day when complete water exchange was carried out. 50 % of the rearing water was also changed everyday.

Experiment 4

Following the failure to determine optimum feeding density of *P. redivivus* in the previous experiment, *P. indicus* larvae were reared to PZ2/PZ3 stage on

the live mixed algae (*T. chuii/ S. costatum*) before the experiment was commenced. Stage PZ2/3 larvae (85 % PZ2) were stocked in 2-l flasks at a density of 75 l^{-1}. Control larvae were fed 25 cells μl^{-1} *T. chuii* and 35 cells μl^{-1} *S. costatum* until mysis 1 (M1) when newly hatched five *Artemia* ml^{-1} were introduced to the culture along with algae until PL stages. Algae ceased totally at M2 stage in this treatment. *P. redivivus* (with no algal co-feed) was offered to the larvae at 15, 30, 45, 60 nematodes ml^{-1}. These nematodes were enriched by replacing 50 % of the corn oil with capelin fish oil to improve fatty acid profile of the nematodes. 50 % of the rearing water was exchanged everyday when larval lengths (10-13 larvae) were measured. The larvae were, however, counted every other day when complete water exchange was performed. Growth rates (between day 1 and day 5) and survival rates (between day 2 and day 5) of the larvae were compared using two-way ANOVA with days as a covariate (General Linear Model) in Minitab statistical package. Growth and survival at metamorphosis were also compared using one-way ANOVA with appropriate pairwise comparison tests (Tukey's and Scheffé's pairwise comparison tests) following Bartlett's test for homogeneity of variances.

Experiment 5

The size effect of the nematodes, *P. redivivus,* on growth and survival of *P. indicus* during larval development was investigated in this experiment. Small (529 ± 226 µm mean length) and large size nematodes (1016 ± 222 µm mean length) were obtained from the AGC. These nematodes were lipid-enriched (with cod liver oil) and pigmented as explained in Chapter 4. The following feeding regimes were used;

(1) Large nematodes throughout larval stages to PL stage (Large),

(2) Small nematodes throughout larval stages to PL stage (Small),

(3) Small nematodes until PZ3/M1 stage and large nematodes thereafter until PL stage (S/L),

(4) Mixed size nematodes all through larval stages (Mixed).

Nematode concentration (ml^{-1} day^{-1}) was increased from 30 (PZ1 and PZ3 stages) to 45 (M1-M2 stages) and finally to 60 (M3-PL stages) during larval culture of *P. indicus*. Nematodes were used within 2-3 days of receipt from the AGC. Growth and survival of larvae were assessed from three replicates for each treatment. Larvae were counted and measured everyday when complete water exchanges were performed. Larval growth and survival data were compared using two-way ANOVA with days as a covariate during protozoeal stages (PZ1-PZ3/M1 or day 2 - day 6) and mysis stages (M1-PL or day 6 - day 9). Larval growth rates and mortality rates were derived from the statistical outputs. Larval growth, as increase in total length, and survival (%) at M1 and PL1 stages were also compared using one-way ANOVA followed by Scheffé's and Tukey's tests after the data was checked for homogeneity of variances using Bartlett's test.

Experiment 6

This experiment was conducted in an attempt to determine optimum feeding density of nematodes by feeding the larvae once or twice a day with the same amount of prey. For this purpose, *P. indicus* larvae (PZ1) were stocked in 2-l experimental flasks at a density of 100 larvae l^{-1}. The nematode *P. rediviv*us (enriched with cod liver oil and pigmented as in the previous experiments) was fed to the larvae at 30 and 50 nematodes ml^{-1} between PZ1 and PZ3/M1 stages. These concentrations of nematodes were introduced into the culture as either one meal or two meals a day (half the ration each time). Generally larvae were fed once in the morning (9.00-11.00 h) and once at night (22.00-24.00 h). Growth and survival were assessed in three replicates everyday when complete water exchanges were performed during protozoeal stages (PZ1-PZ3/M1).

At PZ3/M1 stage, the experiment was terminated and the larvae were stocked into 2-l experimental flasks at 75 larvae l^{-1} in two replicates to assess optimum feeding density of nematodes during mysis and early PL

stages. Hence, three concentrations of nematodes (60, 80 and 100 ml^{-1}) were fed to the larvae in two meals a day (half ration each time) one in the morning and one at night (as during protozoeal stages). Nematode cultures were regularly supplied by the AGC and were used to feed the larvae in 1-2 days of receipt from the company. Survival data during protozoeal and mysis stages did not fit in GLM, hence were excluded in statistical calculations. Growth data was analysed by two-way ANOVA with days as a covariate (GLM) between day 2 and day 6 (PZ1-PZ3/M1), and between day 6 and day 9 (M1-PL1).

RESULTS

Caridean larvae

Palaemon elegans

This experiment was conducted to see if carnivorous larvae of *P. elegans* could be reared on nematodes. Table 3 shows that from the beginning of the experiment the nematode fed larvae demonstrated inferior survival and growth compared to the *Artemia* fed controls. The nematode fed larvae survived only one day longer than the starved control.

Larval growth on the nematodes was also similar to that of the starved larvae. By day 8, both these latter treatments had 100% mortality as opposed to the *Artemia* fed larvae which sustained over 90% survival at this period. The similar trends in growth and survival of the nematode fed and starved larvae together with the observations of larval guts suggest that *P. elegans* larvae were unable to capture the nematodes. This may be due to the physical size and mobility of *C. elegans*. Prior to total mortality, the larvae on the nematodes developed only to *Z3* by day 8, while the *Artemia* fed controls were at stage Z5. The latter larvae continued normal development to postlarvae (PL) on day 12 with 88.5 % survival.

Table 3. Survival (%) of *P. elegans* larvae fed either *C. elegans*, *Artemia* or starved from Z1 stage. Each value represents a mean ± s.d. (n=2).

Days	*C. elegans*	*Artemia*	Starved
	Survival (%)		
0	100.00 ± 0.00	100.00 ± 0.00	100.00± 0.00
2	96.00 ± 2.83	98.50 ± 2.12	94.50 ± 4.95
4	47.50 ± 3.54	94.50 ± 0.71	21.50 ± 0.71
6	3.50 ± 10.61	92.50 ± 0.71	1.50 ± 0.71
8	1.50	91.50 ± 2.12	0.00
	Total length (mm)		
0	3.12 ± 0.03	3.12 ± 0.03	3.12 ± 0.03
2	3.18 ± 0.06	3.72 ± 0.05	3.15 ± 0.04
4	3.19 ± 0.01	4.37 ± 0.05	3.15 ± 0.01
6	3.20 ± 0.06	4.75 ± 0.37	3.17 ± 0.10
8	3.25	5.76 ± 0.03	-

Macrobrachium rosenbergii

Experiment 1

The purpose of this experiment was to investigate whether a commercial fresh water prawn species *M. rosenbergii* could be fed on nematodes during larval development. Growth and survival results of *M. rosenbergii* larvae starved and fed on either *Artemia* or three species of nematodes are summarised in Tables 4a and 4b.

Table 4a. Survival (%) of *M. rosenbergii* larvae starved or fed *Artemia,* and three different species of nematodes at varying concentrations. Each value represents a mean ± s. d. (n=2).

Day	Starved	*Artemia*	*C. elegans*		*S. carpocapsae*	
			(75 ml^{-1})	(150 ml^{-1})	(75 ml^{-1})	(150 ml^{-1})
0	100.00	100.00	100.00	100.00	100.00	100.00
2	99.00±1.73	100.00	100.00	100.00	100.00	100.00
			S. feltiae			
			(150 ml^{-1})	(225 ml^{-1})	(125 ml^{-1})	(175 ml^{-1})
4	97.67±3.21	97.34±2.31	90.67±8.08	90.33±8.62	95.00±3.46	98.67±1.16
6	83.5±3.54	91.00±7.94	14.14±0.00	2.12±0.00	40.00±0.00	44.50±4.95
8	0.00	87.76±6.43	0.00	0.00	0.00	0.00

By day 4-6, it was clear that the prawn larvae were unable to grow and develop on any of the nematode species irrespective of the feeding level. On day 6, all starved and nematode fed larvae remained at stage Z2 while 90 %

of the controls had progressed to Z4 stage. A supply of the larger, lipid enriched *S. feltiae* on day 4 did not reverse the gradual decline in larval survival. By day 8, all starved and nematode fed cultures collapsed. The *Artemia* fed controls continued normal development and started to develop into the PL1 stage with a 67 % final survival on day 24.

Table 4b. Larval total length (mm) of *M. rosenbergii* starved or fed *Artemia*, and three different species of nematodes at varying concentrations. Each value represents a mean ± s.d. (n=2).

Day	Starved	*Artemia*	*C. elegans*		*S. carpocapsae*	
			(75 ml^{-1})	(150 ml^{-1})	(75 ml^{-1})	(150 ml^{-1})
0	2.210±0.03	2.210±0.03	2.354±0.02	2.360±0.03	2.310±0.01	2.348±0.01
2	2.296±0.02	2.338±0.02	2.363±0.02	2.380±0.01	2.360±0.01	2.349±0.01
			S. feltiae			
			(150 ml^{-1})	(225 ml^{-1})	(125 ml^{-1})	(175 ml^{-1})
4	2.299±0.02	2.939±0.01	2.368±0.01	2.384±0.02	2.367±0.01	2.352±0.01
6	2.308±0.01	3.503±0.16	-	-	-	-
8	-	4.453±0.06				

Experiment 2

A similar trend to that observed in the first experiment was established with poor larval development and total mortality by day 8 on the nematode (see Table 5). On day 6, 80 % of the *Artemia* fed controls were at stage Z5 while 95% of the nematode fed larvae remained at stage Z2. Observations of the larvae (Z1) under the microscope revealed that they were unable to capture enough prey. In case the larvae were not fed on a sufficient quantity of nematodes, another batch of larvae were fed on pigmented *P. redivivus* at densities of 200 and 300 ml^{-1}. These larvae died on the 6th day of culture. Some larvae were observed under a binocular microscope in petri dishes. It was found that the larvae were creating a current towards their mouth resulting in congregation of nematodes around the larvae under static conditions in petri dishes. However, the prawn larvae were generally not able to grasp and hold the nematodes in the manner that *Artemia* nauplii were captured. Although use of pigmented nematodes did show that some larvae had ingested nematodes into the gut, the majority remained empty.

Figure 1a

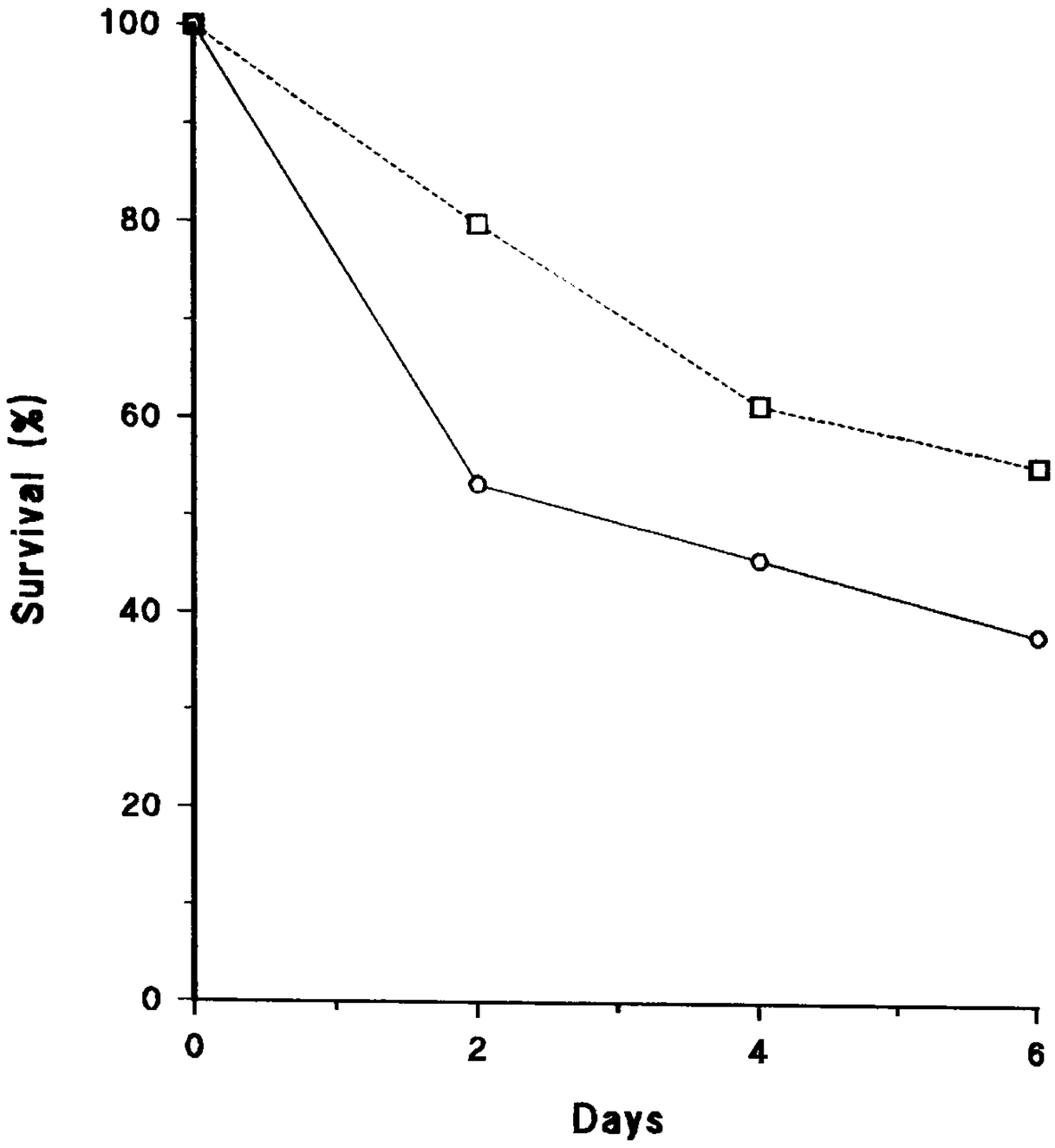


Figure 1b

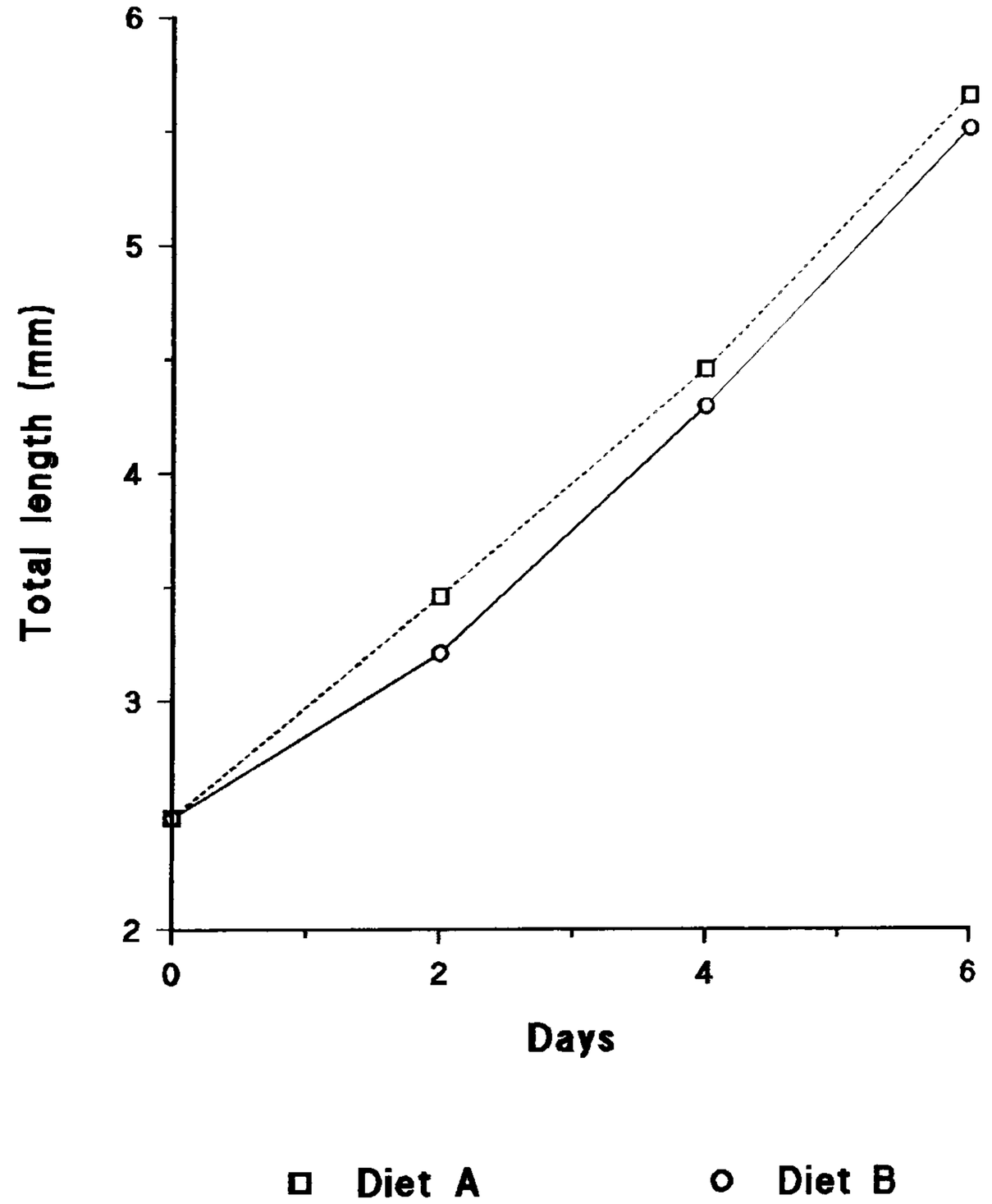


Figure 1a, b. Survival (%) and growth (mm) of *P. indicus* larvae fed on Diet A (algae/nematode) and Diet B (algae/*Artemia*) from PZ2/PZ3 to PL stages. Each value is a mean (n=2).

Table 5. Survival (%) and total length (mm) of *M. rosenbergii* larvae starved, fed *P. redivivus*, and *Artemia* from Z1 stage. Each value is a mean ± s. d. (n=2).

Days	Starved	*Artemia*	*P. redivivus*
		Survival (%)	
0	100	100	100
2	100.00	100.00	100.00
4	100.00	97.50 ± 1.50	99.00 ± 1.00
6	18.00 ± 2.00	95.00 ± 3.00	69.00 ± 3.00
8	0.00	93.50 ± 3.50	0.00
		Total length (mm)	
0	2.83 ± 0.02	2.83 ± 0.02	2.83 ± 0.02
2	2.91 ± 0.09	2.97 ± 0.01	2.92 ± 0.03
4	2.85 ± 0.04	3.28 ± 0.05	2.91 ± 0.04
6	2.85 ± 0.06	3.70 ± 0.10	2.77 ± 0.05
8	-	4.16 ± 0.02	-

Penaeid larvae

Experiment 1

This experiment was conducted in an attempt to replace *Artemia* with the nematode *P. redivivus* during mysis and early PL stages of *P. indicus*. The larvae reared on the nematode *P. redivivus* demonstrated normal growth, development and survival through PZ2/3 and mysis stages to postlarvae (see Figures 1a, b and Table 6). Although nematodes promoted higher larval survival (56 %) and growth (5.7 mm TL) than those (38 %, 5.5 mm TL) fed *Artemia*, differences were not significant at 0.05 probability level (Tables 7a, b). By day 6, 75% of the larvae (PZ2/3 at stocking) had attained the PL1 stage on the nematode (Diet A) while only 60 % of those reared on *Artemia* reached PL1 stage (Diet B). The PL's fed nematodes were considerably paler in coloration than *Artemia* fed larvae but appeared equally healthy and active. During the trial the larval faecal strings produced by feeding on the nematodes were clearly observed.

Table 6. Growth and survival data for Figures 1a, b. Each value is a mean ± s.d. (n=2). Diet A (algae/nematode) and Diet B (algae/*Artemia*).

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

Table 7a. 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 7b. 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	37	16.250	0.439			
Total	38	16.294				

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

Experiment 2

In contrast to *P. redivivus*, *C. elegans* did not support survival and growth beyond day 3. This species of nematode caused high mortality as early as day 2 of the experiment resulting in only 66% survival. Due to technical problems during the size grading of *P. redivivus* cultures, it was not feasible to clean fresh supplies of this nematode species for the remainder of the trial. Consequently, an inappropriate amount of the nematode culture medium entered the larval culture flasks during subsequent feeds following day 2 of the trial when survival of the larvae on this diet was still 87%. This resulted in immediate fouling of the shrimp larvae, which hindered their ability to feed and moult. High mortality resulted and only 10% of the larvae achieved

the PZ3 stage with some larvae moulting to M1 before the cultures collapsed completely. At this time mean survival and total length (mm) of control larvae was 80.5 % and 3.98 mm (all at M1 stage).

Experiment 3

In this experiment adequate feeding levels of nematodes from 30 to 60 ml^{-1} were investigated for PZ1-PZ3/M1 stages of *P. indicus* larvae. Similar to the previous trial, the larvae consumed the nematode and displayed very high survival and growth during early days of the culture (Table 8). However, at PZ3 stage, the larvae became weaker and only a small quantity of them passed into stage M1. Some larvae at this stage were observed to be fouled hence the experiment was terminated at PZ3/M1 stages. Due to low concentrations of the nematodes it was not possible to clean the nematode cultures properly during the experiment.

Table 8. 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).

Days	30 nematodes (ml^{-1})	40 nematodes (ml^{-1})	50 nematodes (ml^{-1})	60 nematodes (ml^{-1})	Control
			Survival (%) ± s.d. (n=3)		
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) ± s.d. (n=3)		
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

Following the failure to determine adequate feeding density of the nematode *P. redivivus*, as food for *P. indicus* larvae in the previous experiment, four different nematode densities (15, 30, 45 and 60 nematodes ml^{-1}) and a control diet were fed to the larvae from stage PZ2/PZ3 to PL1. These nematodes were enriched with 50% fish oil (capelin oil) to ensure sufficient

quantity of essential fatty acids (EFA). Figures 2a and 2b show that the nematode *P. redivivus* was a suitable substitute for *Artemia* nauplii as food for *P. indicus* larvae from stage PZ2/3 to PL1. Nematode fed larvae demonstrated normal growth and survival from PZ2/PZ3 and mysis stages to postlarvae in this preliminary trial. These postlarvae were, however, considerably paler in coloration in comparison to those fed *Artemia*. Survival rate of the larvae fed on the nematode concentrations (15-60 ml^{-1}) were not significantly ($P>0.05$) different from each other (Tables 11a, b). Comparison of the final survivals at metamorphosis showed that the control diet (algae/ *Artemia*) gave a significantly lower survival (66 %) compared to those obtained from the nematodes which ranged from 83 % to 89 % (see Table 9). Irrespective of the feeding levels, the nematode diets also gave significantly lower mortality rates than the control diet (2.74 % day^{-1}). Highest larval growth rate (0.526 mm day^{-1}) was achieved at a density of 60 nematodes ml^{-1} (Tables 12a, b). However, there was no significant difference in larval growth rates between 30, 45 and 60 nematodes ml^{-1} ($P>0.05$). The lowest nematode concentration (15 ml^{-1}) gave significantly inferior growth rate (0.463 mm day^{-1}), than higher levels (see Table 9). Control diet (mixed algae/ *Artemia*) displayed significantly better growth rate (0.534 mm day^{-1}) but higher mortality rate (2.74 % day^{-1}) compared to the nematode diets (see Table 9).

Larvae fed on 15 nematodes ml^{-1} attained the lowest total length (4.44 mm) whereas those fed 60 nematodes ml^{-1} had a final length of 5.15 mm at metamorphosis. When the experiment was terminated, only 37 % of the larvae reared on 15 nematodes ml^{-1} were at PL1 stage, whereas at 60 nematode ml^{-1} the larvae were at 78 % PL1 and 10 % PL2 stages (Table 9). These results suggest that faster larval development is occurring with an increase in the food concentration from 15 to 60 nematodes ml^{-1}. Larvae fed higher nematode concentrations (45-60 ml^{-1}) attained PL stage one day earlier than lower nematode concentrations (15-30 ml^{-1}). The best feeding levels appeared to

Figure 2a

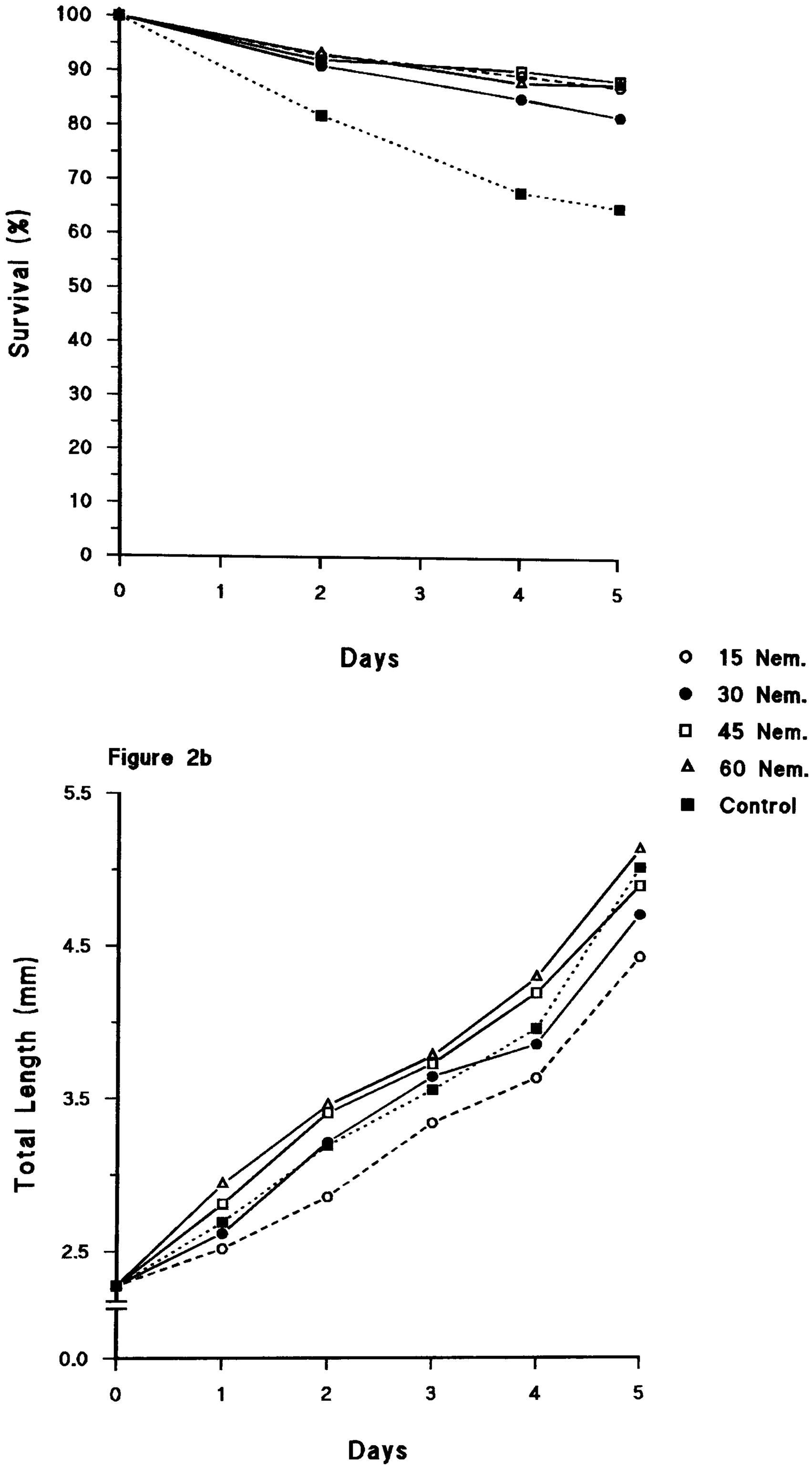


Figure 2b

Figure 2a, b. Survival (%) and growth (mm) of *P. indicus* larvae fed various nematode concentrations (per ml) from PZ2/3 to PL stages. Each value is a mean (n=3).

be between 45 and 60 nematodes ml^{-1} throughout the experiment. Growth and survival data obtained in the present experiment are summarised in Table 10.

Table 9. Growth rate (mm day^{-1}), mortality rate (% day^{-1}), final survival (%), total length (mm) and composition of larval stages of *P. indicus* reared on various density of nematodes (ml^{-1}) and a control diet from PZ2/3 to PL stages. Treatments with the same superscripts are not significantly different (P>0.05). Values for survival and growth are means ± s.d. (n=3).

Diets	Survival at PL1 (%)	Mortality rate (% day^{-1})	Growth at PL1 (mm)	Growth rate (mm day^{-1})	Larval stages at PL (%)
15 nem	88.22^{a} ±4.69	0.850^{a}	4.44^{c} ±0.02	0.463^{c}	54% M2, 20% M3, 26% PL
30 nem	82.67^{a} ±5.29	1.443^{a}	4.72^{b} ±0.03	0.486^{b}	6% M2, 57% M3, 37% PL1
45 nem	89.34^{a} ±1.34	0.517^{a}	4.91^{ab} ±0.05	0.500^{b}	13% M3, 87% PL1
60 nem	88.67^{a} ±2.91	1.023^{a}	5.15^{a} ±0.03	0.526^{b}	12% M3, 78% PL1, 10% PL2
Control	65.78^{b} ±4.91	2.739^{b}	5.03^{ab} ±0.04	0.534^{a}	36% M3, 61% PL1, 3% PL2

Table 10. 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 11a. 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	Ns
Repl*Days(Diets)	10	116.21	116.21	11.62	0.82	0.619	
Error	15	213.64	213.64	14.24			
Total	44	3472.12					

Table 11b. 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	63.09	0.000	
Days		-3.943	0.689	-5.73	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 12a. 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
Days	1	376.940	377.596	377.596	3999.20	0.000	
Diets	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					

Table 12b. 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 (mm day^{-1})	Coeff.	Stdev.	t-value	P	Significant
Constant		2.16935	0.02632	82.44	0.000	
Days		0.501808	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

Survival

(a) PZ1-PZ3/M1

Figure 3a shows survival of *P. indicus* larvae reared on various feeding regimes during protozoeal stages. Comparison of the larval survival indicated that there were significant ($P<0.05$) differences in mortality rates for larvae fed different nematode diets (Tables 15a, b). Larvae fed mixed size nematodes showed significantly lower mortality rate (4.12 % day^{-1}) in comparison

to other treatments. There was no significant difference in the mortality rate of larvae fed on either large or small nematodes (see Table 13). Comparison of larval survivals at M1 stage (Table 14) indicated that only the S/L nematode diet had a significantly lower survival (38.33 %) in comparison to the other nematode feeding regimes ($P<0.05$). No significant difference in survival at M1 stage was found between the larvae fed either small, large or mixed size nematode feeds.

Table 13. Comparisons of mortality rate (% day^{-1}) and growth rate (mm day^{-1}) of *P. indicus* larvae fed various nematode 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
Mixed	4.117^{a}	2.084	0.680	0.439
Large	6.983^{b}	1.534	0.669	0.411
Small	8.467^{b}	0.567	0.659	0.390
S/L	8.500^{b}	2.784	0.672	0.376

(b) M1-PL1

Figure 3b displays survival of the larvae during mysis and early PL stages. Comparison of survival using two-way ANOVA demonstrated that (Table 16a, b) mortality rate of larvae fed the nematode diets did not significantly ($P>0.05$) differ from each other during mysis and early PL stages (Table 13). Percentage survival of postlarvae fed mixed (53%), large (44.33%) and small nematodes were significantly ($P>0.05$) different from each other. S/L nematodes gave significantly lower survival (29.67%) than all other treatments ($P<0.001$) (Table 14). Survival data for Figures 3a, b is summarised in Table 19a.

Growth

(a) PZ1-PZ3/M1

Growth rates of larvae fed on the nematode diets (Figure 4a) were not significantly ($P>0.05$) different during protozoeal stages (Tables 17a, b). Larval

growth rates between PZ1 and PZ3/M1 stages ranged from 0.659 to 0.680 mm day^{-1} (see Table 13). Comparison of larval total lengths at M1 indicated that mixed size and large nematodes promoted significantly ($P<0.05$) better larval total lengths than the other nematode feeds at this stage (Table 14). Small nematodes promoted significantly smaller larval size (4.12-4.14 mm) at M1 stage ($P<0.05$) than large nematodes.

Table 14. 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).

Diets	Survival (%)		Total length (mm)		Duration (days)	
	M1	PL1	M1	PL1	to M1	to PL1
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

(b) M1-PL1

Figure 4b displays growth of the larvae during mysis and early PL stages. Larval growth rates ranged from 0.376 to 0.439 mm day^{-1} between M1 and PL stages (see Table 13). Comparison of larval growth rates (Tables 18a, b) show that that there is no significant difference between larval growth rates, which ranged between 0.376 and 0.439 mm day^{-1}, on different nematode feeds during these stages. Larvae fed small nematodes through all larval stages showed the smallest size at metamorphosis ($P<0.05$). There was no significant difference in the total length of PL's fed on mixed or large size nematodes (Table 14). Growth data for Figures 4a, b is shown in Table 19b. Irrespective of the experimental feeding regimes, all the larvae developed into M1 stage on day 6 and started to metamorphose into PL1 stage on day 8-9.

Figures 3a, b. Survival (%) of *P. indicus* larvae fed 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. Each value is a mean from three replicates.

Figures 4a, b. 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 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. Each value is a mean from three replicates. Each replicate contains measurements of at least 10-14 larvae.

Figure 3a

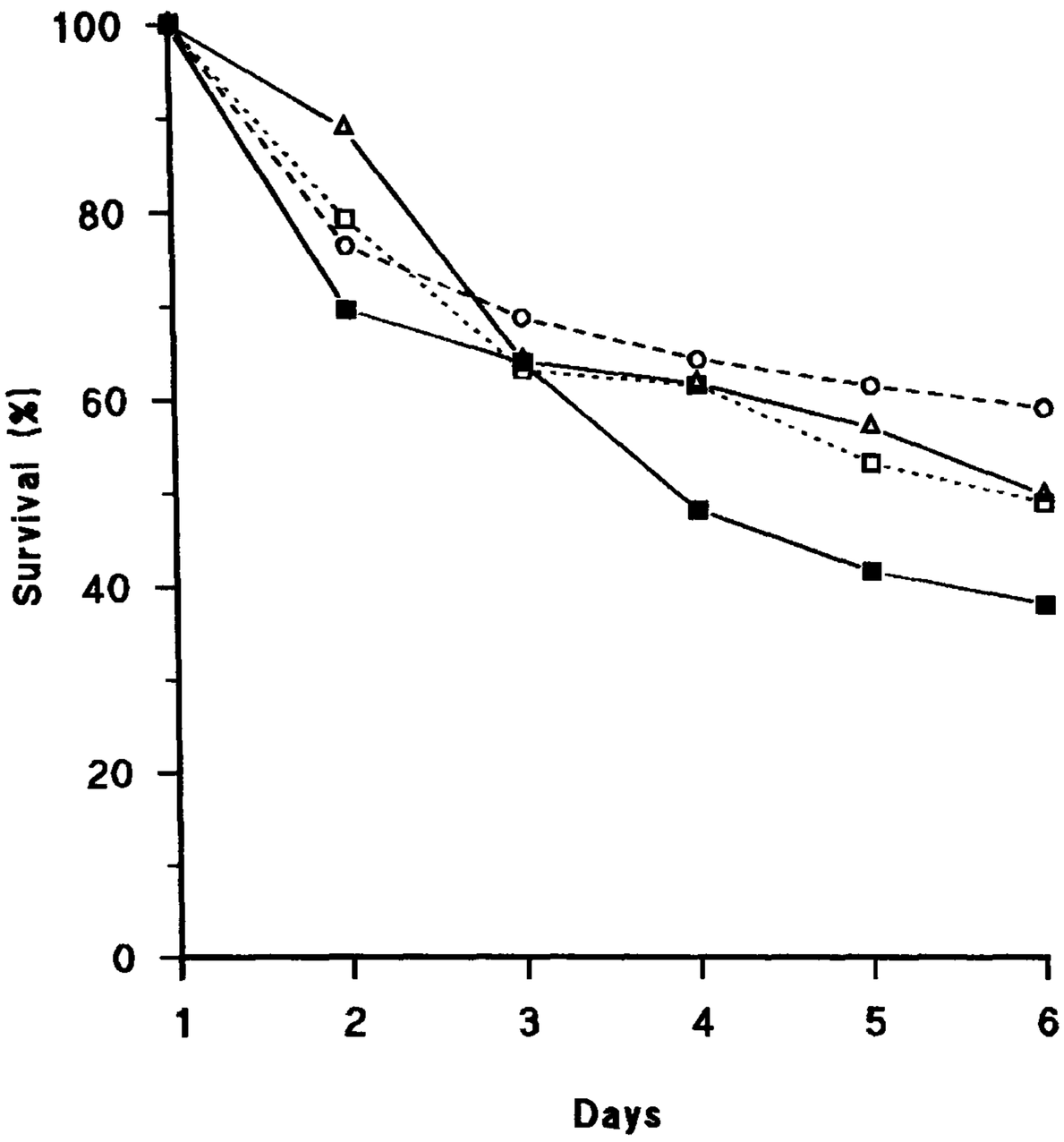


Figure 3b

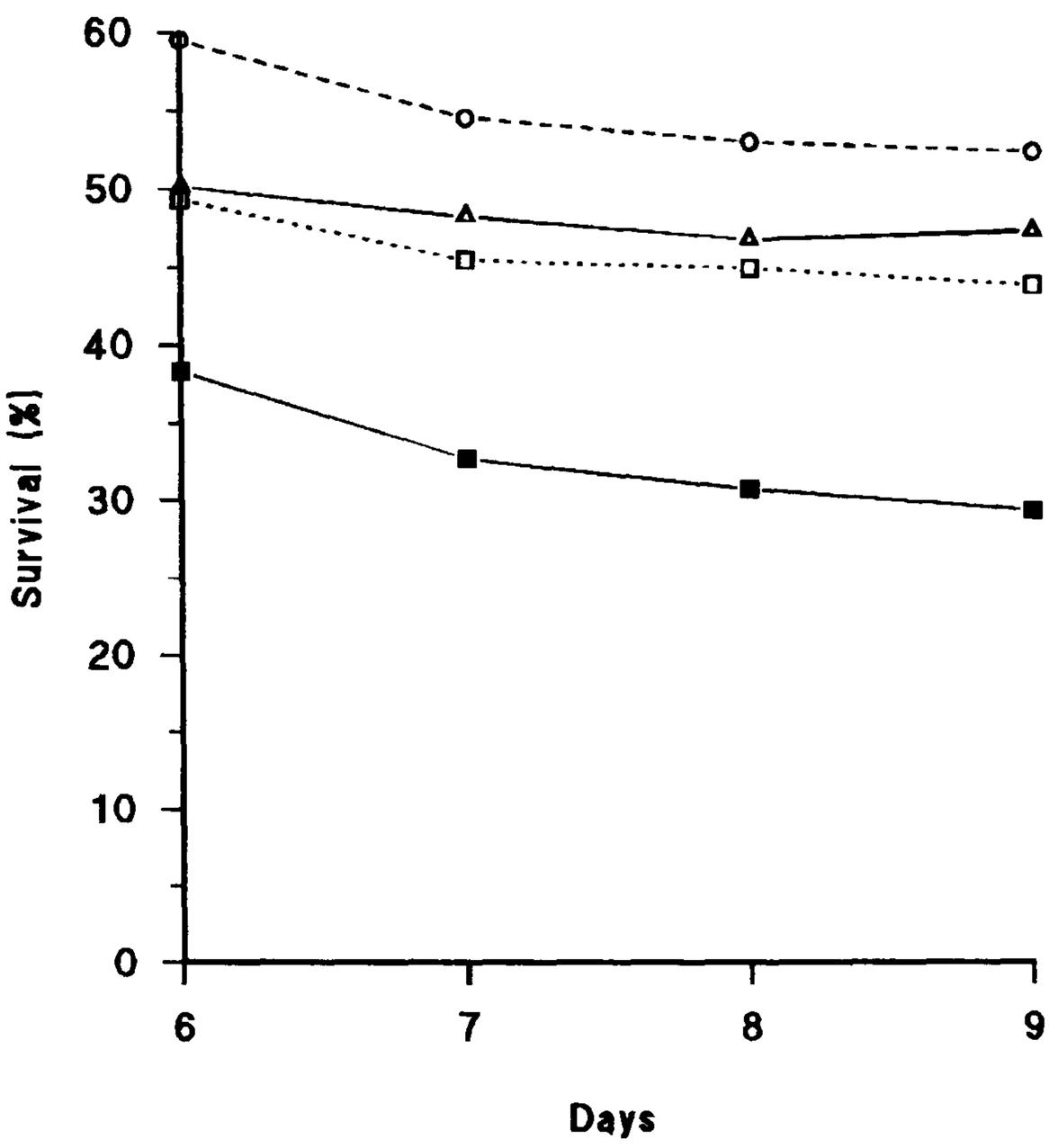


Figure 4a

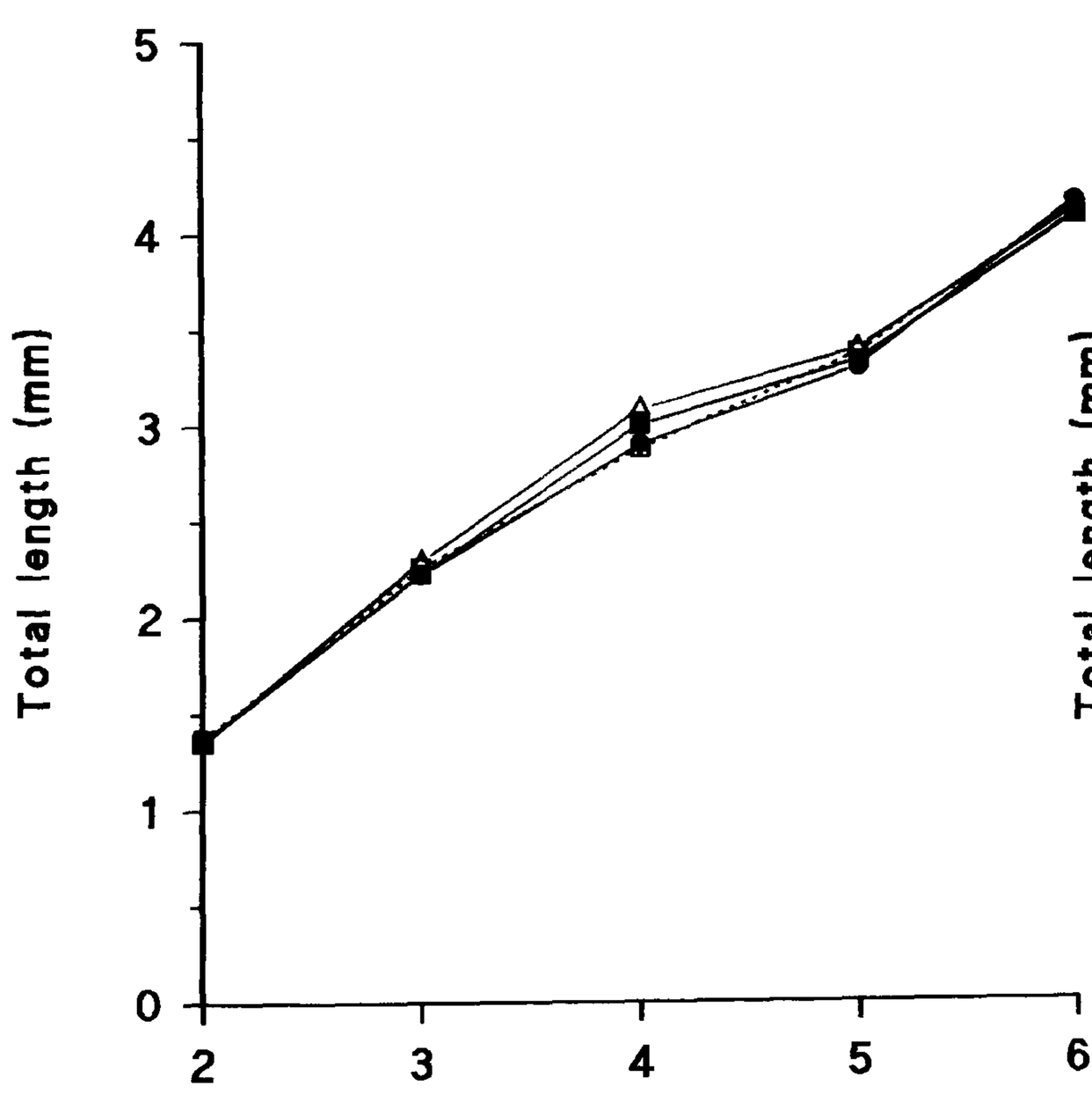


Figure 4b

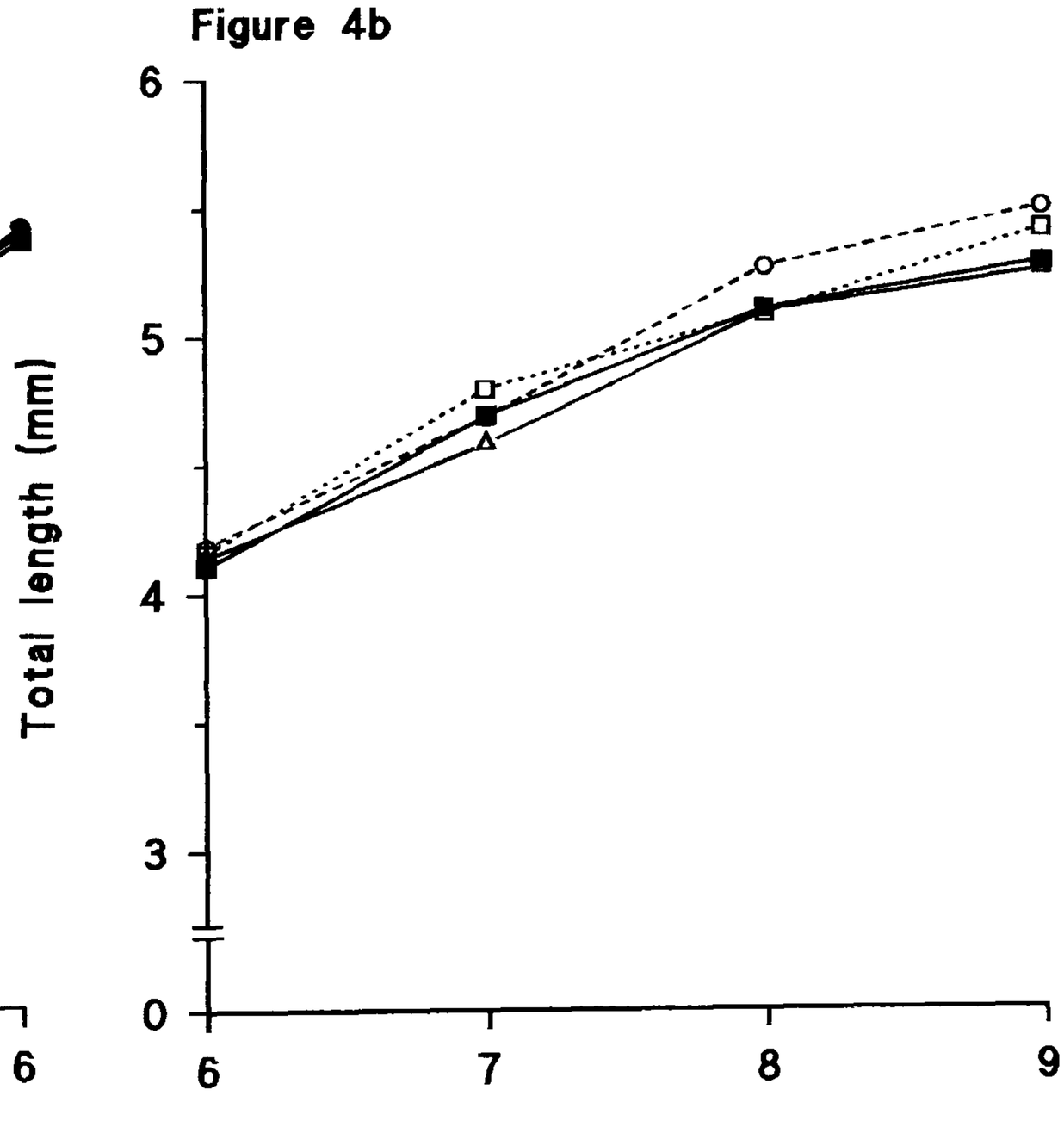


Table 15a. Two-way ANOVA with days as covariate on survival (%) of *P. indicus* on various feeding regimes from PZ1 (day 2) to PZ3/M1 (day 6) stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Diets	3	1705.37	247.93	82.64	1.83	0.158	
Days	1	5908.17	5908.17	5908.17	131.15	0.000	
Repl(Diets)	8	283.52	329.29	41.16	0.91	0.516	
Diets*Days	3	381.45	381.45	127.15	2.82	0.052	Ns
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 15b. Comparison of mortality rates (% day^{-1}) from PZ1 to PZ3/M1.

Term	Mortality rate (% day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
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 16a. Two-way ANOVA with days as covariate on survival (%) 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	Signifi-cant
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(Diets)	8	136.92	108.63	13.58	0.38	0.920	
Diets*Days	3	39.39	39.39	13.13	0.37	0.776	Ns
Repl*Days(Diets)	8	134.78	134.78	16.85	0.47	0.862	
Error	24	852.45	852.45	35.52			
Total	47	4469.81					

Table 16b. Comparison of mortality rate (% day^{-1}) from PZ3/M1 to PL stages.

Term	Mortality rate (% day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
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	Ns
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 17a. Two-way ANOVA with days as covariate on total length (mm) 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	Signifi-cant
Diets	3	0.696	0.073	0.024	0.46	0.709	
Days	1	594.687	593.676	593.676	1.1E+04	0.000	
Repl(Diets)	8	0.628	0.051	0.006	0.12	0.998	
Diets*Days	3	0.075	0.073	0.024	0.47	0.705	Ns
Repl*Days(Diets)	8	0.159	0.159	0.020	0.38	0.932	
Error	637	33.293	33.293	0.052			
Total	660	629.538					

Table 17b. Comparison of the growth rates (mm day^{-1}) from PZ1 to PZ3/M1.

Term	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
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	Ns
Large	0.669	-0.00057	0.01081	-0.05	0.958	Ns
S/L	0.659	-0.01122	0.01092	-1.03	0.305	Ns
Small	0.672	0.00211	0.01087	0.19	0.846	Ns

Table 18a. 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	Signifi-cant
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(Diets)	8	1.5205	0.3600	0.0450	0.47	0.879	
Diets*Days	3	0.4108	0.4321	0.1440	1.50	0.215	Ns
Repl*Days(Diet)	8	0.4176	0.4176	0.0522	0.54	0.825	
Error	578	55.6492	55.6492	0.0963			
Total	601	182.436					

Table 18b. Comparison of growth rates (mm day^{-1}) from PZ3/M1 to PL stages.

Term	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
Constant		2.21780	0.07551	29.37	0.000	
Days		0.40448	0.01137	35.58	0.000	
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
S/L	0.390	-0.01392	0.02018	-0.69	0.490	Ns
Small	0.376	-0.02789	0.01932	-1.44	0.149	Ns

Table 19a. Survival (%) data for Figures 3a, b. 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 19b. Growth data (mm) for Figures 4a, b. 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

Experiment 6

(a) PZ1-PZ3/M1

Survival

This experiment was conducted to see whether there would be any advantage of feeding *P. indicus* larvae (during protozoeal stages) with higher nematode concentrations and/or more than one meal a day. Survival of larvae fed various nematode feeding regimes between PZ1 and PZ3/M1 stages are shown in Figure 5a and Table 21a. Since survival data did not fit in the GLM, no statistical analyses were performed. The nematodes supported high survivals (99-100 %) during the first three days of culture. On the 4th day, however, larvae fed once a day on 30 and 50 nematodes ml^{-1} started to show higher mortalities compared to larvae fed twice a day with the same rations. Larvae fed once a day appeared to be dirty and fouled between day 4 and day 6. When the experiment was terminated on day 6, larvae fed 30 nematodes ml^{-1} twice a day showed the highest survival (71 %) compared to the other feeding regimes. Lowest survivals were found in the larvae fed once a day at 30

(21.17 %) and 50 nematodes ml^{-1} day^{-1} (52.67 %). Feeding the larvae at higher nematode concentrations (50 ml^{-1}) even at two distribution times a day was not successful.

Growth

Growth of larvae on the nematode feeding regimes are shown in Figure 5b. Tables 20a, b show that larval growth rates were significantly ($P<0.001$) affected by the nematode feeds during protozoeal stages. Highest larval growth rates were obtained from the larvae reared on 30 nematodes ml^{-1} in two meals (0.667 mm day^{-1}) and 50 nematodes ml^{-1} once a day (0.671 mm day^{-1}). Larvae fed 30 nematodes ml^{-1} once a day showed significantly a lower growth rate (0.566 mm day^{-1}) (see Table 20b). Total lengths (mm) of larvae fed on 30 nematodes in one meal and two meals a day at M1 stage (day 6) were 3.64 mm and 4.12 mm respectively (Table 21b). Larval total lengths on 50 nematodes in one meal and two meals at M1 stage were, however, 4.16 mm and 3.94 mm.

(b) M1-PL1

Survival

This experiment was performed to test any benefit of increasing the nematode density from 60 to 100 ml^{-1} with two distribution times a day for *P. indicus* mysis and early PL stages. Figure 6a shows that survival of the larvae fed various densities of nematodes at M1 showed a gradual decline during the first two days of the culture. During this period, larvae fed 60 nematodes ml (in two meals a day) consistently displayed higher survival compared to those fed higher nematode concentrations (80-100 nematodes ml^{-1}). On day 9, however, all larval cultures had high mortalities irrespective of nematode concentrations. The larvae appeared to be fouled, inactive and weak at metamorphosis. The reason for this is thought to be a possible contamination of the nematode culture used on day 8. These nematode cultures

Figures 5a, b. Survival (%) and growth (mm TL) of *P. indicus* larvae fed on 30 or 50 nematodes ml^{-1} in one (x1) or two distribution times (x2) a day from PZ1 to PZ3/M1 stages. Each value is a mean from two replicates.

Figures 6a, b. 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. Each value is a mean from two replicates.

Figure 5a

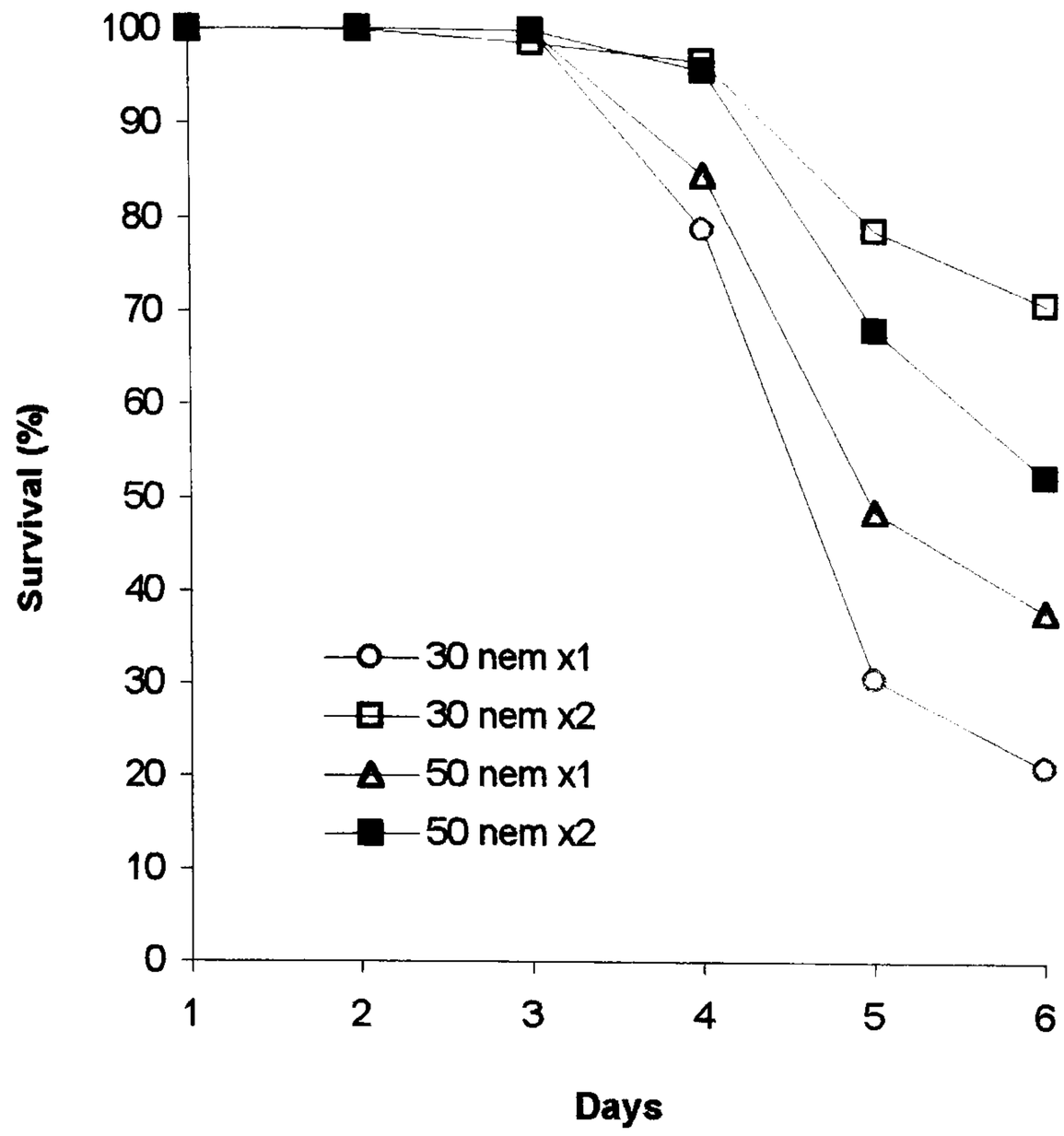


Figure 5b

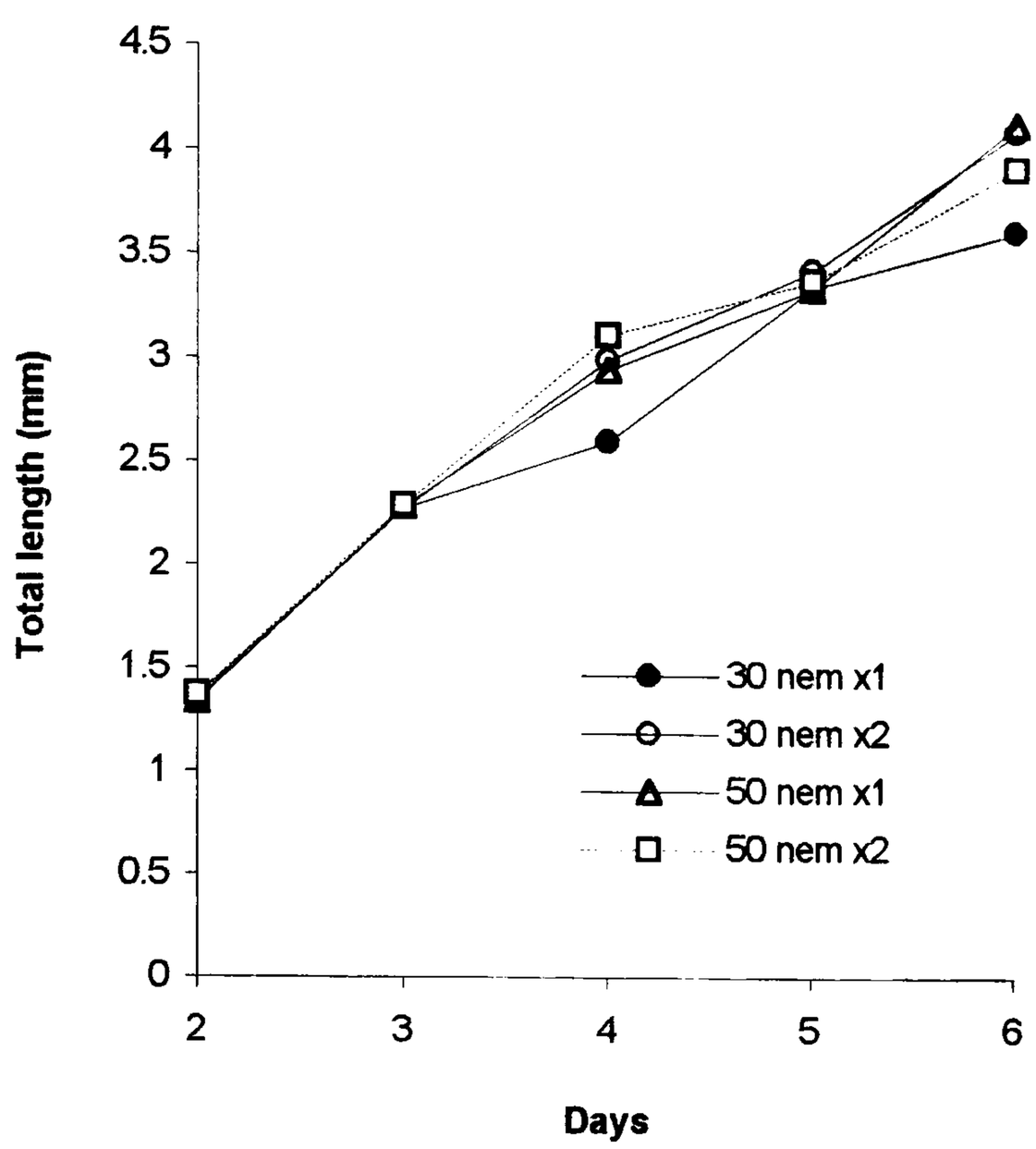


Figure 6a

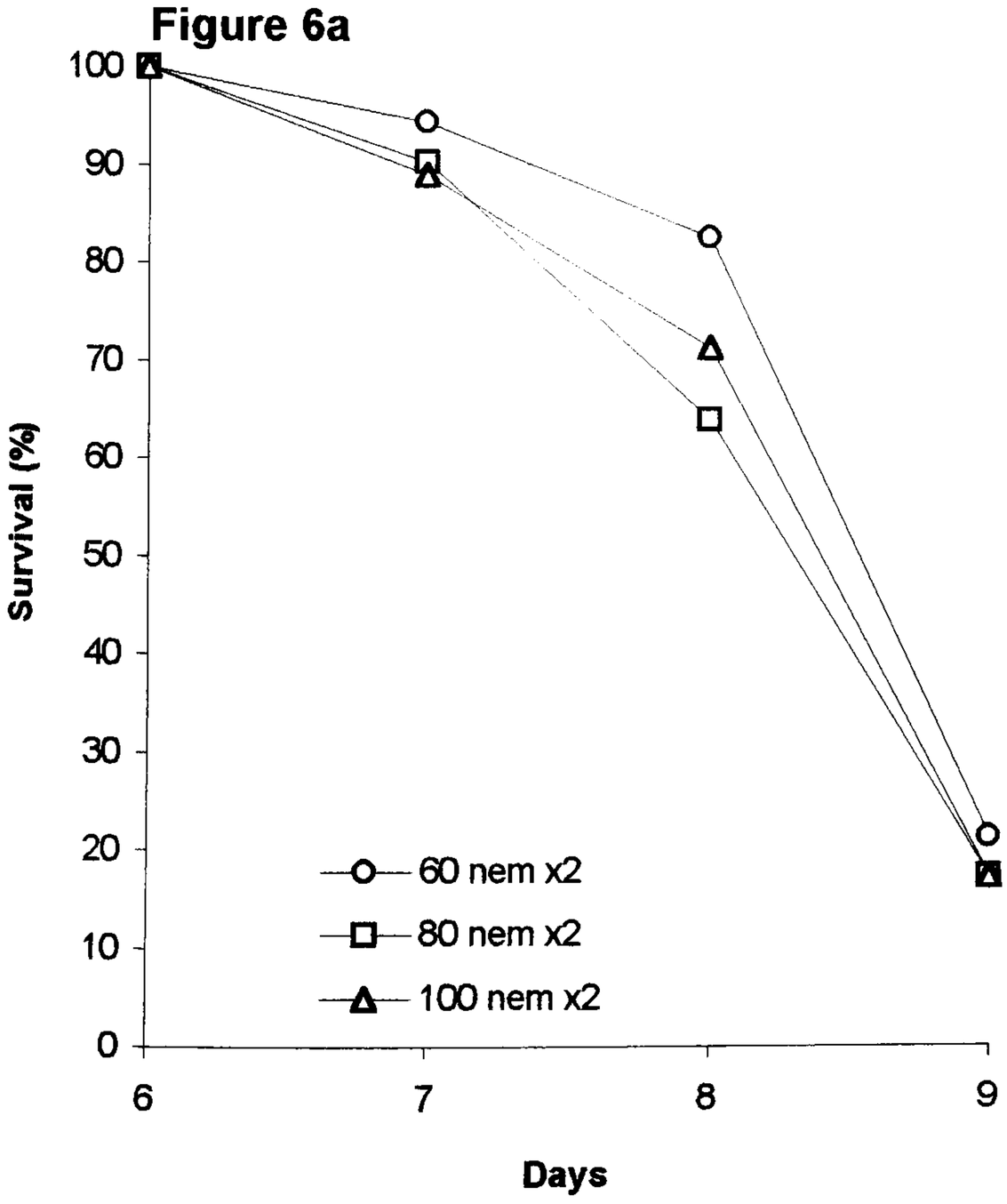
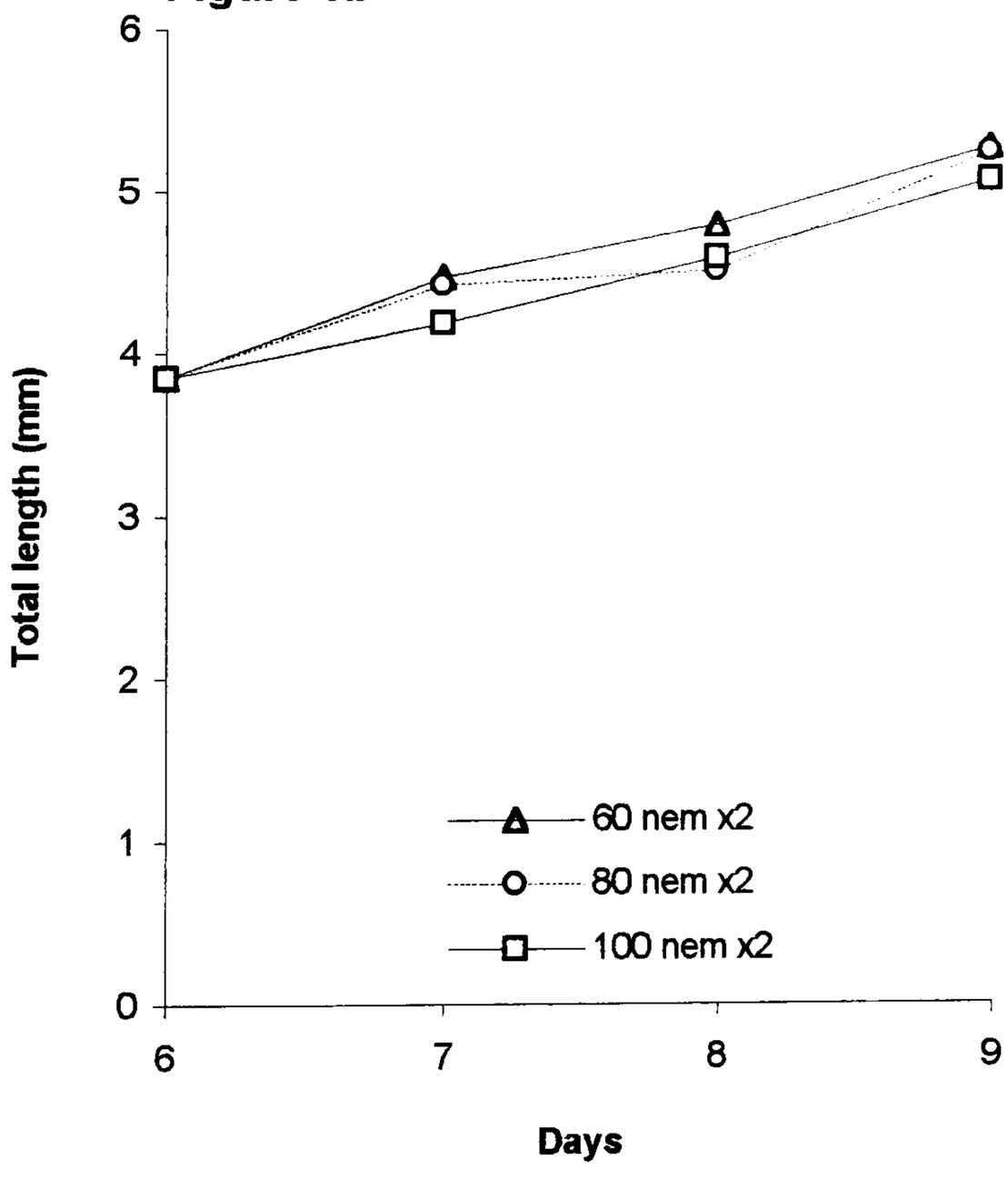


Figure 6b



had stayed at room temperature for two days until they were received from the supplier company (AGC).

Growth

Figure 6b exhibits growth of the larvae fed on 60, 80 and 100 nematodes ml^{-1} in two meals a day from M1 to PL1 stages. Comparison of the slopes of growth lines indicated that there was no significant ($P>0.05$) effects of the nematode feeding regimes on larval growth rates of *P. indicus* between M1 and PL1 stages (see Figure 6b and Tables 22a, b). Larval growth rates on 60, 80 and 100 nematodes ml^{-1} were 0.405, 0.418 and 0.446 mm day^{-1} respectively. Total lengths at metamorphosis on 60, 80 and 100 nematodes were 5.29, 5.26 and 5.09 mm respectively (Table 23).

Table 20a. 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	Signifi-cant
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	0.000	***
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 20b. Comparisons of larval growth rates (mm day^{-1}) from PZ1-PZ3/M1 stages.

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

Table 21a. Survival data (%) for Figure 5a. Each value is mean ± s. d. (n=3).

Days	30 nem x1	30 nem x2	50 nem x1	50 nem x2
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	48.67 ± 29.75	68.33 ± 22.99
6	21.17 ± 5.20	71.17 ± 15.33	38.00 ± 25.64	52.67 ± 29.61

Table 21b. Growth (mm) data for Figure 5b. Each value is a mean ± s. d. (n=3).

Days	30 nem x1	30 nem x2	50 nem x1	50 nem x2
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 ± 0.04
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

Table 22a. Two-way ANOVA with day 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	Signifi-cant
Diets	2	1.7571	0.4950	0.2475	2.46	0.088	
Days	1	25.3691	25.3067	25.3067	251.12	0.000	
Repl(Diets)	3	0.3675	0.1827	0.0609	0.60	0.613	
Diets*Days	2	0.0398	0.0414	0.0207	0.21	0.814	Ns
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 22b Comparisons of larval growth rates (mm day^{-1}) from M1 to PL1 stages.

Term	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
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 23. Survival (%) and growth data (mm TL) for Figures 6a, b. Each value is a mean ± s. d. (n=2).

	Survival (%) ± s.d.			Total length (mm) ± s.d.		
Days	60 nem x2	80 nem x2	100 nem x2	60 nem x2	80 nem x2	100 nem x2
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	4.43±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

DISCUSSION

Caridean larvae

Caridean larvae hatch as zoea (Z) and directly feed on live zooplankton, mainly *Artemia* nauplii until metamorphosis. Continuous attempts to replace *Artemia,* which may constitute up to 60 % of hatchery costs (Menasveta et al., 1984), with live rotifers (Lovett and Felder, 1988) and artificial diets (Jones et al., 1975; Deru, 1990) have failed. For the first time, live nematodes were used as a total *Artemia* replacement to rear the carnivorous caridean larvae, *P. elegans* and *M. rosenbergii*. Growth and survival results revealed that the larvae of these two caridean shrimp species are unable to grow and survive on the nematodes. Normal larval survival and development in the control treatment (*Artemia* fed larvae) indicated that experimental procedures were appropriate for the culture of the larvae. A preliminary experiment with the native prawn *P. elegans* showed that larvae of this species were not capable of catching and ingesting nematodes. It was not possible to see any ingested prey in the gut of the larvae of this species. As a result, the larvae either starved or when fed on the nematodes survived only until day 7-8 (Z3 stage) using their internal energy sources. At this time the larvae fed *Artemia* were already at Z5 stage with over 90 % survival (see Table 3).

Similarly, the present experiments with the freshwater prawn *M. rosenbergii* demonstrated that the nematodes were not suitable as food for these larvae. Despite high nematode concentrations (75-150 nematodes ml^{-1}) used in the first experiment, larval guts remained empty. A larger nematode species *S. feltiae* fed at even higher concentrations (150-225 ml^{-1}) did not improve survival and also caused a deterioration in water quality. Therefore, complete mortality in both starved and nematode fed larvae occurred on day 7-8 while larvae fed *Artemia* had an 88 % survival at this time (see Table 4a). Even at lower feeding rates (10-30 ml^{-1}) with *P. redivivus* (Experiment 2),

which were not associated with deterioration in water quality, the *M. rosenbergii* larvae once again displayed poor survival and growth similar to those starved control (see Table 5). Complete larval mortality in starved larvae and those fed the nematodes occurred on day 7-8 of the culture. In the case that the larvae might have been fed at insufficient prey levels, some larvae were fed on 200-300 nematodes (*P. redivivus*) ml^{-1}. Again, these larvae died on day 6 indicating failure of the cultures was not due to inadequate feeding rates. Microscopic observations showed that *M. rosenbergii* larvae, like *P. elegans*, were incapable of capturing and ingesting enough nematodes to survive and develop. Using pigmented nematodes (with astaxanthin) in petri dishes, it was possible to see some larvae with ingested pink nematodes in the guts. Yet, most of the larval guts remained empty. During larval sampling from 2-l flasks, larval guts with the ingested nematodes had never been witnessed.

The present results suggest that failure of carnivorous caridean larvae to capture sufficient nematodes may be due to the cylindiroconical body shape (without appendages) and movement or size of the nematodes. The nematodes did not contain any toxic or noxious substances as the larvae were not killed earlier than the starved ones and also some larvae with ingested nematodes were observed. Inappropriate nutritional content of the nematodes seems unlikely since nematodes have been proved to be perfectly adequate as food for various penaeid species such as *P. aztecus*, *P. setiferus*, *P. vannamei* (Wilkenfeld et al., 1984; Biedenbach, 1989) and *P. indicus* (the present study). Studies with caridean larvae in Chapter 6 revealed that these larvae do not readily feed on artificial diets particularly during their early stages (Z1-Z4) when they require easily available and digestible feed such as *Artemia*.

Unlike penaeid larvae, these caridean larvae do not use filter feeding strategies and rely on chance-encounter to capture food particles or prey. Once

the contact has been made with food items both chemical and textural stimuli become important (Moller et al., 1979). Carnivorous *H. gammarus* larvae have been reported to capture food non-selectively but ingestion depends on chemical cues and they ingest only nutritious particles (Kurmaly et al., 1990). Similar observations were made when *M. rosenbergii* and *P. elegans* were fed on artificial diets particularly during early larval stages (See Chapter 6). The late larvae (Z5/6 onwards) of both *P. elegans* and *M. rosenbergii* were able to survive on artificial particles which ranged in size from 100-400 µm (Chapter 6). Although size range (length) of the nematode species used in the present study ranged from 400-950 µm in length and 37-65 µm in diameter, no larvae were observed to capture the nematodes in the 2-l experimental glass flasks.

Since carnivorous larvae are unable to locate their food items and are chance-encounter feeders, their ability to discriminate and avoid low nutritious value food may enable them to ingest not only nutritious items but also those that are easily digestible (Kurmaly et al., 1990). As caridean larvae have limited digestive capabilities as demonstrated in Chapter 6, the chemical and textural stimulus and relatively hard and thick cuticle of the nematodes (Hofsten et al., 1983) might have affected the larval food ingestion and possibly digestion. As discussed in the later sections, unlike carnivorous larvae the omnivorous *P. indicus* larvae were, however, able to successfully ingest and digest the nematodes.

Penaeid larvae

Nematodes as microalgae and *Artemia* replacement

As with other penaeids, *P. indicus* larvae require microalgae during protozoeal stages and an animal food source during later stages (Emmerson, 1980). The purpose of the present study was to completely eliminate the use of microalgae and *Artemia* during larval culture of *P. indicus*. Results of Chapter 1 revealed that this penaeid species can be successfully reared on

mixed algae (*T. chuii/S. costatum*) during herbivoral stages and plus five *Artemia* nauplii ml^{-1} during mysis and early PL stages with or without the presence of microalgae. The potential of the nematode *P. redivivus* as an alternative food for *P. indicus* was first tested on mysis stages before an adequate feeding density and the possibility of a total replacement from the first feeding stage were attempted. The experimental data suggested that *Artemia* nauplii can be successfully replaced totally with the nematode *P. redivivus* in rearing *P. indicus* larvae during mysis and early PL stages. When PZ2/PZ3 stage of *P. indicus* larvae were fed on the algae/ nematodes until M2/M3 and on nematodes solely during subsequent stages (until PL1), the larvae displayed equal growth, development and survival compared to the algae/*Artemia* control treatment (see Figures 1a, b). Larvae fed the nematodes at PL1 appeared to be active and healthy but considerably paler than those fed on *Artemia*.

Following determination of the suitability of the nematode *P. redivivus* as food for mysis stages, several experiments were conducted to feed *P. indicus* larvae as early as the first feeding stage (PZ1). High mortalities were observed when the larvae (PZ1) were fed solely on the nematode regimes (*P. redivivus* and *C. elegans*) in the preliminary trials. This was attributed to an addition of inappropriate amount of nematode culture medium into the larval cultures as cleaning of the nematodes was not feasible during the experiment (see Experiments 3 and 4). This resulted in a deterioration of culture water and an immediate larval fouling which hindered the feeding and moulting processes. For similar reasons, early trials to determine adequate feeding density of the nematodes also collapsed (Experiment 4). These results demonstrate that nematode cultures for successful use in penaeid larval culture must be free of the growth media.

A successful complete replacement of the conventional live diets in larval culture of *P. indicus* was demonstrated through all larval stages to metamor-

phosis (Experiment 5). When PZ1 stage larvae were fed on the non-enriched (lipid) nematodes in the absence of microalgae, survival up to 53 % was obtained until PL1 stage with 5.51 mm TL (Figures 3a, b). Microscopic observations and long faecal strings produced by the larvae have indicated that the PZ1 larvae were capable of capturing, ingesting and digesting the nematodes. Wilkenfeld et al., (1984) also report that it was possible to successfully culture *P. aztecus*, *P. vannamei* and *P. setiferus* on the nematode *P. redivivus* from PZ1 to PL1 stage without algae. In their study, the three penaeid species responded to the live nematode feeds differently.

The present data shows that despite the nematodes supporting slower larval growth rate and development in *P. indicus*, they generally promote superior larval survivals in comparison to the algae/ *Artemia* feeding regimes (see Figures 1a, 2a). For example, larvae fed *P. redivivus* displayed significantly higher survivals (83-89 %) than those fed algae/*Artemia* (66 %) from PZ2/ PZ3 to PL stages (Figure 2a). Wilkenfeld et al., (1984) also found inferior growth and larval development in *P. aztecus*, *P. setiferus* and *P. vannamei* on the sole nematode regimes compared to live algae/*Artemia* or algae/ nematodes. However, larval growth rate may be improved depending on which stage an animal food is added into the culture. For example when *P. indicus* larvae were fed on mixed algae (*T. chuii/ S. costatum*) until PZ2/PZ3 stages and then the nematodes were added into the culture, larvae fed the nematodes displayed equal growth, survival and development compared to those fed *Artemia* nauplii (Figure 2b). Wilkenfeld et al., (1984) suggested that nematodes should be added to the culture medium already containing algae when the penaeid larvae were at PZ1 stage in order to get metamorphosis and dry-weight growth equal to those fed algae with *Artemia* added at M1 stage. Biedenbach et al., (1989) showed that it was possible to get equivalent growth, survival and larval development in *P. vannamei* when the larvae fed on an algae/nematode diet (starting from PZ1 stage) an algal feed or a standard algae/*Artemia* regime until metamorphosis.

The present results obtained in the laboratory experiments showed that the nematode, *P. redivivus*, is a suitable alternative diet for *P. indicus* larvae both as an algae and *Artemia* replacement in small culture vessels and should be seriously considered for use in penaeid hatcheries after their potential is tested also in large tanks. Live nematodes offer several practical advantages over algae and *Artemia*; for example, unlike *Artemia*, they do not consume algae, never grow too large to be consumed by the larvae, and can survive in sea water up to 72 h (Fontaine et al., 1992 cited in Biedenbach et al., 1989). They can be cultured easily on various types of media. Culture in liquid medium allows them to be produced in mass quantities in large vessels suitable for commercial purposes (Fisher, pers. comm.). They have very high reproductive rates. Their nutritive value is high and comparable to *Artemia* (Kahan et al., 1980; Léger and Sorgeloos, 1992). Their biochemical composition can be manipulated by loading the alimentary canal with growth factors (Kahan et al., 1980) such as lipids, HUFA, and pigments as demonstrated by Rouse et al., (1992) and in the present study (Chapter 4).

It is well known that microalgae and *Artemia* used predominantly in penaeid cultures are inconsistent in their nutritional quality (Watanabe et al., 1983; Léger et al., 1986) and may cause a considerable risk of disastrous effects on productivity in commercial penaeid larviculture (Wilkenfeld et al., 1984). The nutritional value of the nematodes may be kept relatively consistent in comparison to microalgae and *Artemia*. They may be produced inexpensively by using cheap ingredients in their culture media. One negative aspect using nematodes, however, may be their high settlement rate which may occur in large vessels. Therefore, it is proposed that when free of contaminating culture medium, the nematodes may provide a more reliable source of nutrients to penaeid larvae in comparison to microalgae and *Artemia*.

Feeding density of nematodes

Wilkenfeld et al., (1984) used 70 nematodes (*P. redivivus*) ml^{-1} to rear *P. aztecus*, *P. setiferus* and *P. vannamei* during larval development. These authors determined the feeding level of nematodes which would give protein equal to that of three *Artemia* ml^{-1}. Biedenbach et al., (1989) studied feeding level of nematodes of *P. vannamei* at different larval stages but with algae present together with the nematodes in the culture system. Results of Experiment 4 (Table 9) showed that among the nematode concentrations tested (15, 30, 45 and 60 nematodes ml^{-1} day^{-1}) on *P. indicus* larvae between PZ2/3 to PL1 stages, the lowest nematode concentrations promoted the lowest growth rate (0.463 mm day^{-1}) compared to those fed higher nematode concentrations. No significant difference was found between the growth rates of the larvae fed on 30, 45 and 60 nematodes ml^{-1} (0.500-0.534 mm day^{-1}). Mortality rate of the larvae fed on these nematode concentrations were also not significantly different from each other ($P>0.05$). All the nematode concentrations supported between 83 % and 89 % larval survival until metamorphosis ($P>0.05$) in comparison to the inferior performance of the control (algae/*Artemia*) feeding regime (66 %). 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 such as 5 to 25 (PZ3), 10 to 50 (M1) or 15 to 100 (M2) nematodes ml^{-1}.

Comparison of large or small nematodes (Figures 3, 4) demonstrated that *P. indicus* larvae do not show any preference for small (529 ± 226 µm) or large size (1016 ± 222 µm) nematodes as the larvae displayed similar growth and survival irrespective of the prey size offered. It seems that mixed size nematodes (natural cultured population) provide a more adequate size range to fast developing penaeid larvae. Observations in petri dishes showed that *P. indicus* larvae (PZ1 stage) were successful in handling individual nematodes until they were consumed completely. A high rate of settlement of nematodes was observed during the present experiments, hence higher

nematode concentrations were investigated in Experiment 6. The results indicated that 30 nematodes ml^{-1}, when given twice a day (15 nematodes ml^{-1} at 09.00-11.00 h and 15 nematodes ml^{-1} at 22.00-23.00 h), promoted a higher survival (71 %) than that 50 nematodes ml^{-1} (53 %) which were also fed in two meals to the larvae between PZ1 and PZ3/M1 stages. Higher nematode concentrations in either one or two meals a day polluted the culture water and caused aggregations of the nematodes in the culture water and on the larvae. Data obtained during mysis stages suggest that nematode concentrations higher than 60 ml^{-1} do not improve growth or survival of *P. indicus* larvae at least between M1 and M3 stages. Therefore, high nematode concentrations are not suggested for the culture of *P. indicus* in the present experimental conditions and concentrations above 60 ml^{-1} in large scale larval rearing may cause considerable losses. On the last day of the trial (Experiment 6), all the nematode feeding regimes caused unexpected high mortalities possibly due to a fungal or bacterial contamination of the nematode cultures. This type of contamination is sometimes observed during the culture of the nematodes (Fisher, pers. comm.). Nevertheless the larvae fed 60 nematodes ml^{-1} in two meals per day promoted consistently better larval survival before the larval cultures were affected by the contamination. Results of Biedenbach et al., (1989) also support the present findings in that concentrations of nematodes from 40 to 200, 25 to 100 or 300 nematodes ml^{-1} produced similar dry weights at PL stage of *P. vannamei*. Therefore, it is proposed that 30 nematodes ml^{-1} during protozoeal stages (PZ1-PZ3), 45 nematodes ml^{-1} during M1-M2 and 60-70 nematodes ml^{-1} between M3-PL1 stages, 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.

CHAPTER 4

THE EFFECTS OF ASTAXANTHIN- AND/OR LIPID-ENRICHED NEMATODES (*Panagrellus redivivus*) ON LARVAL PIGMENTATION, SURVIVAL, GROWTH AND DEVELOPMENT OF *Penaeus indicus*.

Parts of this chapter were presented in 'WORLD AQUACULTURE' '95' San Diego, USA, February 1-4, 1995.

Title: Growth and survival of *Penaeus indicus* larvae fed on the nematode *Panagrellus redivivus* enriched with astaxanthin and various marine lipids.

Authors: Fletcher, D. J., Kumlu, M. and Fisher, C. M.

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INTRODUCTION

The nutritional profile of the nematode, *Panagrellus redivivus* is comparable to that of *Artemia*, containing 40-62 % protein, 15-20 % lipid and 31% carbohydrate (Biedenbach et al., 1989; Léger and Sorgeloos, 1992). The amino acid composition of *Panagrellus* sp., is also reported to be similar to that of *Artemia* (Kahan et al., 1980). Studies have revealed that among highly unsaturated fatty acids (HUFA) 20: 5ω-3 and 22: 6ω-3 are essential for penaeid larvae and may be required in the larval diets (Jones et al., 1979a). It was demonstrated that although *P. redivivus* lacks 20: 5ω-3 in comparison to *Artemia,* neither the nematode nor *Artemia* contain high levels of 22: 6ω-3 (Biedenbach et al., 1989). The lipid content of *P. redivivus* has been improved when grown on a medium containing wheat flour enhanced with fish oil (Rouse et al., 1992). No studies, however, have been conducted to show the effects of the lipid profile enriched nematodes on larval growth, survival and development of penaeid larvae.

The pigmentation level of an aquatic animal may be an important factor affecting its market value and it may also directly indicate the healthiness and quality of an organism (Bird and Savage, 1990; Latscha, 1990; Chien and Jeng, 1992). In the case of intensive shrimp and fish farming, the natural pond pigment sources become insufficient and hence the integument of the animals fades. As a result, carotenoids are conventionally included in the diets of various fish and crustaceans. Carotenoids are the compounds responsible for crustacean pigmentation. Although they can be produced *de novo* by algae, fungi and higher plants, all animals have to rely on the supply of carotenoids from their diet (D'Abramo et al., 1983; Torrissen, 1989; Latscha, 1990; Esterman, 1994). Although the most commonly used carotenoids in commercial diets are cantaxanthin and astaxanthin (Bird and Savage, 1990; Johnson and Ann, 1991), studies with fish and crustaceans have shown that astaxanthin is the most effective pigment (Torrissen, 1989; Yamada et al., 1990; Chien and

Jeng, 1992). It is known that astaxanthin is the major carotenoid, representing 68-98 % of the total carotenoids associated with pigmentation in shrimps (Latscha, 1990; Négre-Sadargues et al., 1993). Aquatic animals are capable of altering dietary carotenoids by oxidation and depositing them in their tissues. Crustaceans can convert various algal carotenoids such as lutein and zeaxanthin and β-carotene to the major pigment, astaxanthin (Latscha, 1990). Yamada et al., (1990) report that carotenoids (cantaxanthin, β-carotene and astaxanthin) are deposited in the tissue of *P. japonicus* mainly as astaxanthin esters.

Apart from their function in pigmentation and as vitamin A precursors, other biological functions of carotenoids are less well known. It has been suggested that carotenoids may influence many physiological functions of aquatic animals. Of particular relevance is the provitamin A activity of carotenoids. Animals are unable to synthesise vitamin A which is essential for a variety of functions including vision, growth, reproduction and resistance to a variety of diseases (Torrissen, 1989). Carotenoids are also involved in gonadal development, maturation, fertilisation and hatching in fish and crustaceans (Meyers, 1977). They are also known to associate with proteins to form carotenoproteins (Cheesman et al., 1967) that influence the stability of proteins and enzyme activities (Britton et al., 1981). Carotenoids are also known to enhance growth, reproduction and fecundity in crustaceans and fishes (Tacon, 1981). Torrissen (1984) reported improved growth rates in alevin of *Salmo salar* when fed diets containing carotenoids. A recent study on the pigmentation of *P. japonicus* juveniles showed that dietary carotenoids may improve survival and growth (Chien and Jeng, 1992). Négre-Sadargues et al., (1993) also showed that astaxanthin and cantaxanthin are stored in the integument and hepatopancreas of *P. japonicus* juveniles improving survival rate. In contrast to these findings, Yamada et al., (1990) showed no notable increase in growth, feed efficiency and daily feed intake of *P. japonicus* reared on diets with and without carotenoids. D'Abramo et al., (1983) also reported that there was no significant effects of the pigments on the

wet-weights of juvenile lobster *Homarus americanus* reared on artificial diets containing various carotenoids. Chien and Jeng (1992) suggested the inclusion of 100 mg astaxanthin per 100 g diet one month before the harvest of *P. japonicus* to get the best pigmentation level. With the exception of one study (Petit et al., 1991), there is almost no information about carotenoid metabolism in penaeid larvae.

Results of Chapter 3 showed that although nematodes promote higher larval survival in *P. indicus*, they give relatively inferior growth and development compared to an algae/*Artemia* feeding regime. The primary objective of this chapter was, therefore, to further improve 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; see Chapter 5) to improve digestibility of the prey (Hofsten et al., 1983). The nematodes which had been enriched with several lipid sources (capelin oil, cod liver oil and marilla oil) to improve their essential fatty acid (EFA) profile, 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. The 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

Experiment 1

This experiment was conducted to determine the effect of the nematode *P. redivivus*, which had been lipid-enriched and pigmented, either alone or to-

gether with an algal co-feed on larval growth, survival and colour of *P. indicus* from PZ1 to PL stages. For this purpose the following diets were used;

(a) Lipid-enriched nematodes (EN),

(b) Non-enriched (NEN),

(c) Lipid-enriched and pigmented nematodes (PEN),

(d) Pigmented and non-enriched nematodes (PNE),

(e) Enriched-nematodes plus 30 cells μl^{-1} mixed algae (*T. chuii /S. costatum*, 1:1) for only 24 h at PZ1 stage (EN/algae),

(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.

Since *P. redivivus* is a terrestrial nematode species, it was thought that it might lack some of essential fatty acids particularly 20: 5ω-3 and 22: 6ω-3 (HUFA) that marine penaeid larvae require in their diets (Jones et al., 1979a). Hence, in some nematode cultures 50 % of the corn oil was replaced with capelin fish oil to enrich the fatty acid profile of the nematodes. Carophyll Pink (Hoffmann La Roche Ltd., Switzerland), which contains 8 % astaxanthin, was added to the nematode cultures in an attempt to improve the pale colour of the larvae normally obtained with non-pigmented nematodes. Any physiological effects of the astaxanthin was also investigated on larval growth and survival of *P. indicus*. 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 (Fisher, pers. com.). The same author suggests that 1.5 % (w/w) Carophyll Pink, which provides 1.43 μg^{-1} astaxanthin g^{-1} dry weight of the nematodes, was the maximum level that can be used in the medium to pigment the nematodes. Densities of 30 nematodes ml^{-1} during protozoeal stages, and 60 nematodes ml^{-1} during mysis stages were used in this experiment.

Studies in Chapter 5 revealed that addition of a low amount of algae into the larval rearing water as a co-feed together with artificial diets improved larval growth and survival of *P. indicus* particularly during protozoeal stages. Thus,

some larvae were fed on 30 cells μl^{-1} of the mixed algae *T. chuii /S. costatum* (1:1) for a period of only 24 h in addition to the normal nematode ration (EN/algae). A complete water exchange was carried out everyday when the larvae were staged and survival-growth data was collected. Growth and survival results were derived from three replicates for each diet. During the experiment it was not always possible to obtain fresh nematodes from AGC.

Since 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 survival (at PL) and growth (total lengths) were also compared using one-way ANOVA and appropriate pairwise comparison tests (Scheffé's test and Tukey's test) following Bartlett's homogeneity test for variances.

Experiment 2

Although enhancement of larval colour was demonstrated, it was not possible to obtain clear and reliable data to suggest any positive effect of the lipid and/or pigment enrichment on larval growth and survival due to irregular supply and dirty cultures of the nematodes in Experiment 2. Therefore, this experiment was repeated to determine the effects of lipid enrichment (with capelin fish oil) and pigmentation on survival and growth of *P. indicus* larvae. All the nematode cultures were used within 1-2 days of receipt from the AGC in this experiment. Three different algal co-feeds along with the nematodes were also tested on larval growth, survival and trypsin activity 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 nematodes (EN)

(c) Pigmented and lipid-enriched nematodes (PEN)

(d) Pigmented and non-lipid-enriched nematodes (PNE)

(e) Lipid-enriched nematodes 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 48 h (EN/Alg2)

(g) Lipid-enriched nematodes plus 15 cells μl^{-1} mixed algae (*T. chuii /S. costatum*, 1:2) for 48 h (EN/Alg3)

(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 when 75 PZ2 stage larvae were sampled for trypsin analysis and dry weight determinations (30 PZ2 for each replicate) in two replicates for each treatment. Each sample was assayed for trypsin activity in at least three replicates as described in General Material and Methods (GMM). Larval body dry weight was also determined using the method described in GMM. Protein, lipid and fatty acid profiles of the nematodes used in the present study were supplied by AGC (Table 13).

Slopes of survival data were 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 (day 1 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 (Scheffé's and Tukey's tests). Larval trypsin activities at PZ2 stage were compared using one-way ANOVA and Tukey's test after the data was checked for homogeneity of variances with Bartlett's test.

Experiment 3

The effects of nematodes (*P. redivivus*) enriched with different lipid sources on larval growth and survival of *P. indicus* were compared in this experiment. All nematode cultures were used within 1-2 days of receipt from the 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 *Artemia* 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. 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. Larval count was also performed daily to determine survival (%) during 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. Protein, lipid and fatty acid profile of the nematodes used as food to rear *P. indicus* larvae in the present experiment were obtained from AGC. 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 be-

tween PZ1-PZ3/M1 stages (day 2-day 6), and M1-PL stages (day 6-day 10). Comparison of larval growth and survival at M1 and PL1 stages were performed using one-way ANOVA followed by Scheffé's and Tukey's pairwise comparison tests after the data was checked for homogeneity of variances using Bartlett's test.

Experiment 4

In Experiment 3, the potential impact of the pigment astaxanthin, in relation to growth and survival, was inconclusive. Consequently, the experiment was repeated using nematodes enriched either with the pigment or the placebo pigment capsules. In this manner, 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).

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 supplied by AGC.

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 pairwise comparison tests were conducted using Scheffé's test for growth and Tukey's test for survival.

RESULTS

Experiment 1

Survival

Figures 1, 2a and 2b show survival and growth of larvae of *P. indicus* reared on various nematode diets and a control diet from PZ1 and PL stages. This experiment demonstrated that complete replacement of live algae and *Artemia* in the culture of *P. indicus* throughout all larval stages to the metamorphosis can be achieved successfully using the nematode *P. redivivus* alone. Since survival data did not fit in the GLM model, logarithmic transformation was carried out before survival rate of larvae reared on different feeding regimes were compared between day 2 and day 10. Table 2a shows that there were significant effects of the treatments on larval survival ($P<0.001$). Lowest log mortality rate ($P<0.001$) was achieved with lipid enriched nematodes plus 30 cells μl^{-1} algal co-feed (EN/algae) from PZ1 to PL1 stage with a final survival of 72 % at PL1 stages (see Tables 2a and 2b). EN and PEN nematodes gave significantly higher mortality rate than non-enriched nematodes.

There was no significant difference between the mortality rate of the larvae fed on the control diet and those reared on PNE nematodes (see Table 1). The pigmented nematode (PEN and PNE) cultures were not as clean in comparison to non-enriched (NEN) and lipid-enriched non-pigmented (EN) nematode cultures. This resulted in fouling of the larvae during culture causing higher mortalities. Comparisons of final survivals at PL stages (day 10) demonstrated that EN/algae supported significantly ($P<0.05$) higher larval survival (72 %) compared to all other feeding regimes (Table 1). There was no significant difference ($P>0.05$) in larval survival on NEN (57 %), PNE (50 %), control (49 %) and EN (42 %) nematodes at PL stages. PEN nematodes, however, displayed the lowest survival (26 %) at metamorphosis (see Figure 1).

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). Values for survival and growth are means ± s.d. (n=3).

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}	$56.67^{b} \pm 2.52$	0.484^{d}	0.328^{c}	$5.10^{c} \pm 0.14$
EN	0.047^{a}	$42.17^{b} \pm 2.25$	0.505^{d}	0.331^{c}	$5.18^{c} \pm 0.14$
PNE	0.026^{c}	$49.67^{b} \pm 1.26$	0.441^{d}	0.349^{b}	$5.01^{c} \pm 0.05$
PEN	0.043^{b}	$26.00^{c} \pm 2.65$	0.560^{c}	0.328^{c}	$5.29^{c} \pm 0.09$
EN/algae	0.018^{e}	$71.50^{a} \pm 3.54$	0.623^{b}	0.304^{d}	$5.68^{b} \pm 0.09$
Control	0.031^{c}	$49.33^{b} \pm 2.25$	0.716^{a}	0.548^{a}	$6.90^{a} \pm 0.13$

Growth

(a) PZ1-PZ3/M1

Daily larval sampling allowed comparison of the effects of the nematode feeds with algae during protozoeal stages (day 1 to day 4) and with *Artemia* during mysis stages (day 4 to day 10). Table 3a shows that there are significant (P<0.001) differences in the growth rate of the larvae on various feeding regimes between PZ1-PZ3/M1 stages. Figure 2a and Table 3b demonstrate that highest larval growth rate (0.72 mm day^{-1}) was achieved with the mixed algae (P<0.001) with a 3.98 mm TL at PZ3/M1 stage (see also Table 1). Growth rate of larvae fed 30 cells μl^{-1} of algae (*T. chuii/S. costatum*, 1:1) for only 24 h together with lipid-enriched nematodes was significantly better (0.62 mm day^{-1}) than the larval growth rate obtained from EN nematodes (0.51 mm day^{-1}). Pigmentation of the nematodes clearly improved the pale colour of the larvae, but did not improve larval growth rate (0.44-0.56 mm day^{-1}) compared to non-pigmented nematode fed larvae (0.48-0.50 mm day^{-1}). Difference in the colouration between the larvae fed pigmented nematodes and non-pigmented nematodes was clearly visible under a microscope as early as at PZ1 stage (24 h fed). The poor performance of the larvae fed PEN nematodes may again have been due to the extremely dirty nematode culture, which may have resulted in serious larval fouling particularly during early

larval stages. Enrichment of the nematodes with capelin oil neither improved survival nor growth rate of the larvae compared to NEN nematodes during protozoeal stages. Larvae fed EN nematodes had relatively high survivals and appeared to be healthy and active at PZ1 and PZ2 stages. At PZ3 stage, however, they started to display an abnormal 'L' shaped body. This might indicate a nutrient imbalance of this nematode diet rather than any environmental problem encountered during larval culture. Improper larval development from PZ3 stage onwards affected the subsequent survival and growth of the larvae fed on the EN nematodes.

Growth

(b) M1-PL

Figure 2b and Tables 4a, b show that larval growth rate (0.55 mm day^{-1}) and final total length (6.90 mm) promoted by *Artemia* were significantly better than all other nematode diets ($P<0.001$) between M1 and PL stages. Growth rate of the larvae which were previously fed algal co-feed for a period of only 24 h at PZ1 stage was significantly lower (0.30 mm day^{-1}) than those on other nematode feeds during mysis stages, although their final TL at PL stages was still significantly greater (5.68 mm). No significant improvements of lipid-enrichment and/or pigmentation of the nematodes on larval growth were found between M1 and PL stages (see Table 1). Growth and survival data obtained in this experiment are shown in Tables 6a and 6b.

Larval development

Table 5 shows that the fastest larval development was achieved with the control feed followed by the EN/algae both during protozoeal and later stages. On day 4, all the larvae fed on algae were at M1 stage whereas only 73 % of those fed EN/algae were at M1 and the remaining 37 % at PZ3 stage. On day 4, 62 % of the larvae reared on PEN were at M1 stage compared to 13 % of those reared on NEN nematodes. At termination of the experiment, almost 35 % of the control larvae were at PL3/4 stages. Among the nematode

Figure 1. Survival (%) of *P. indicus* larvae reared on various feeding regimes from PZ1 to PL stages. Each value is a mean (n=3). (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).

Figures 2a, b. Growth (mm) of *P. indicus* larvae reared on various feeding regimes (a) from PZ1 to PZ3/M1 and (b) from M1 to PL stages. Each value is a mean (n=3). (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).

Figure 1

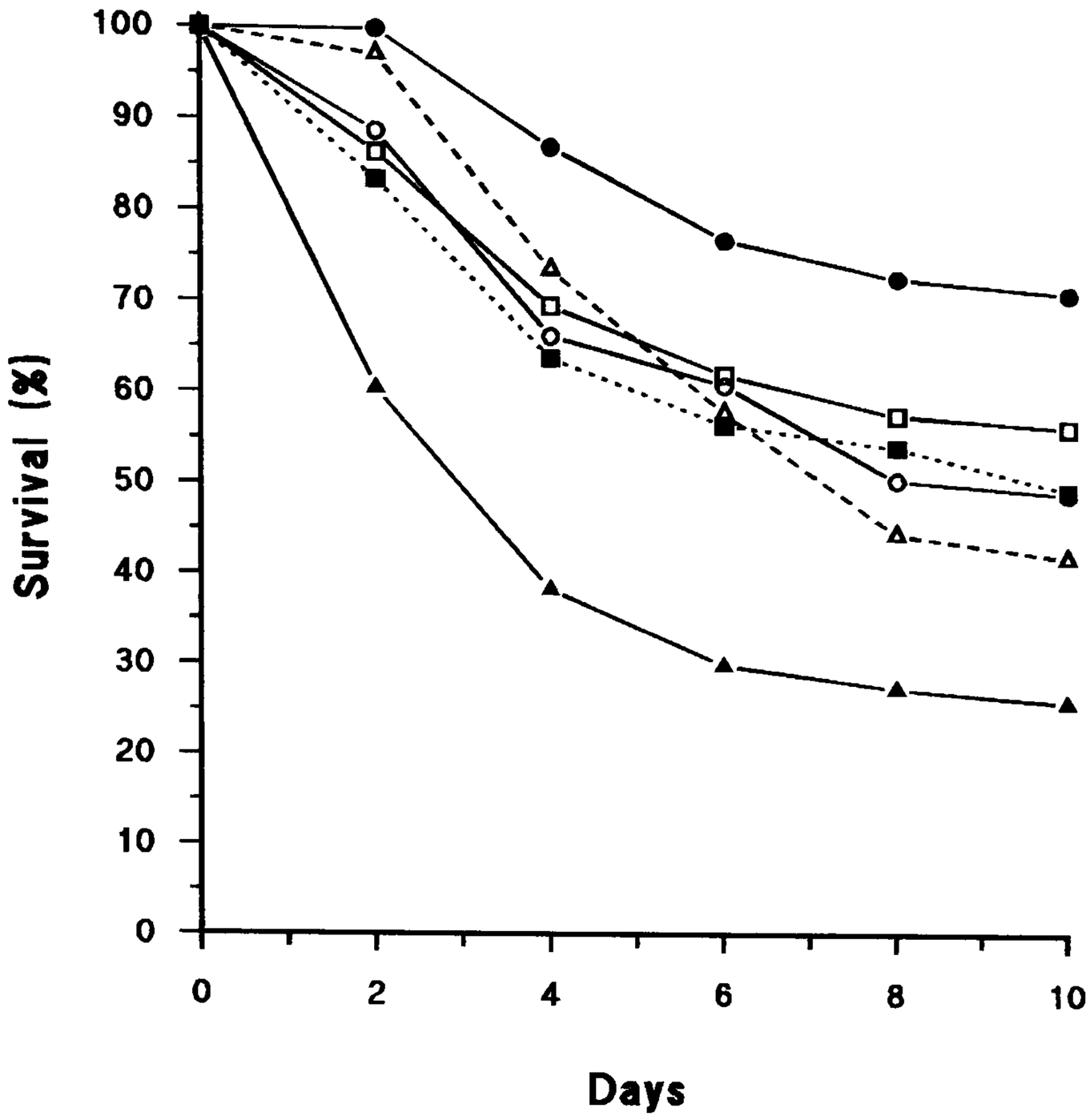


Figure 2a

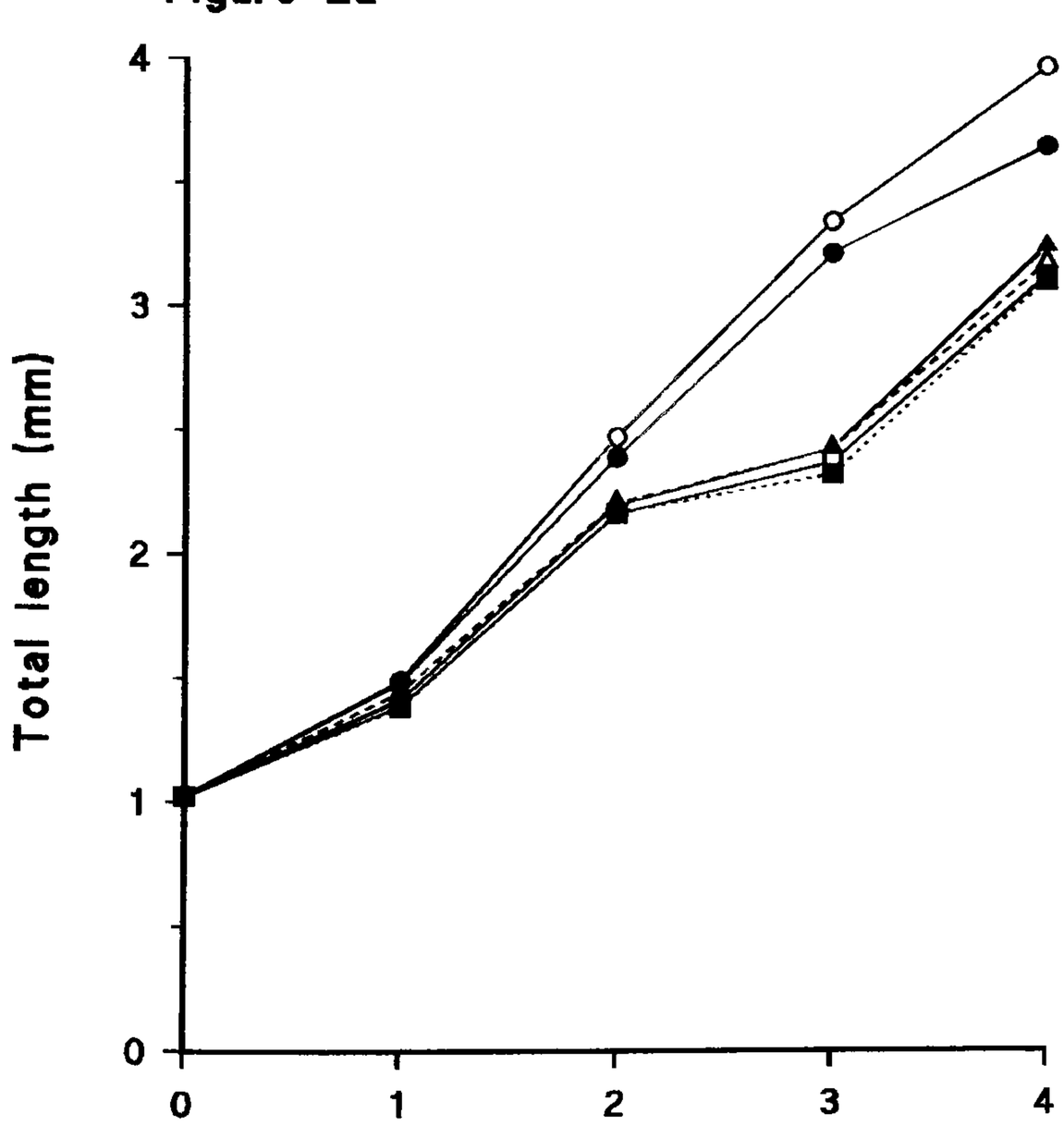
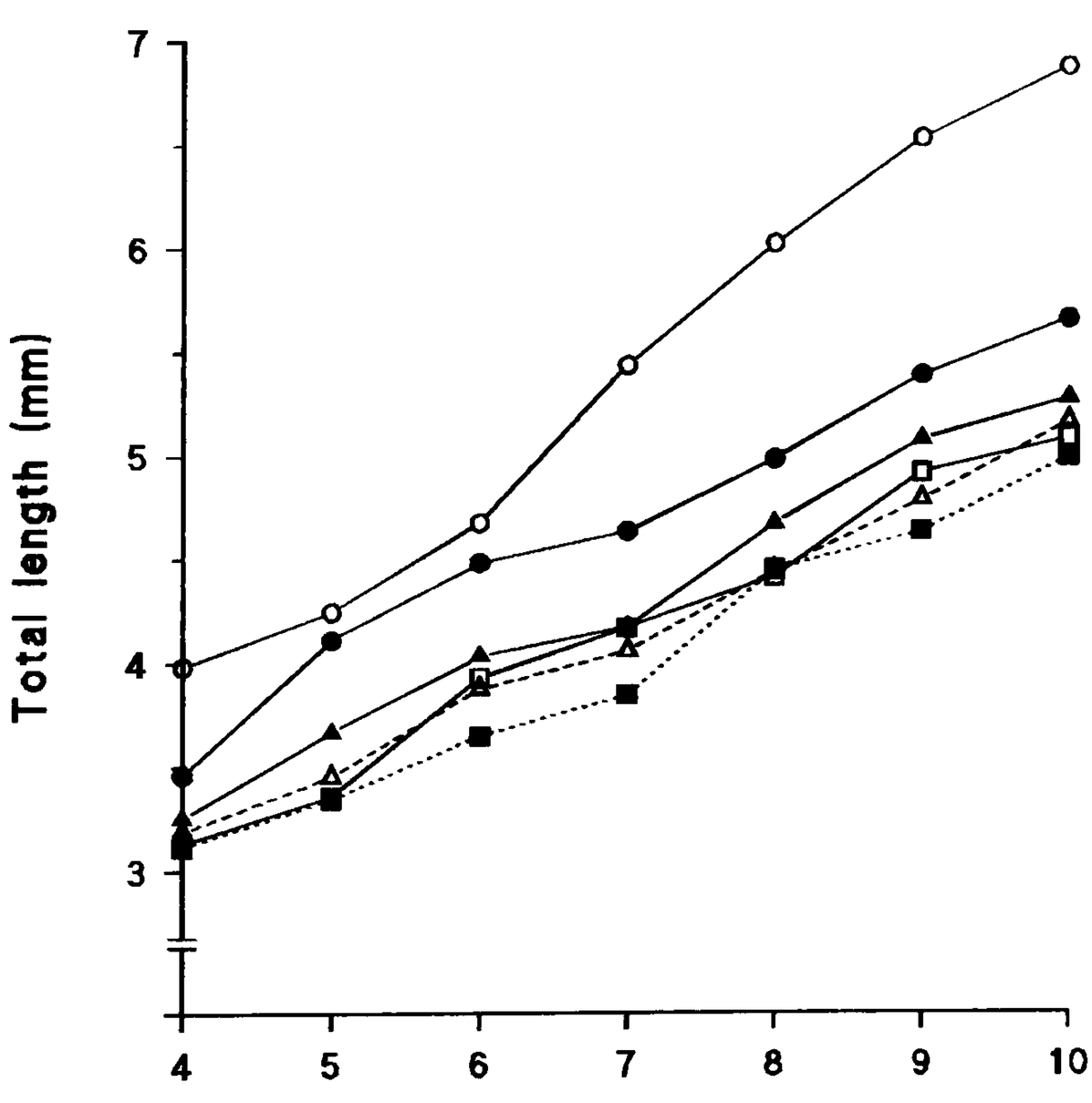


Figure 2b



feeds, EN/algae and PEN diet supported faster development (PL1/PL2) compared to others (M3/PL1) on day 10.

Table 2a. Two-way ANOVA with days as a covariate on log larval survival of *P. indicus* from PZ1 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	0.009512	0.010791	0.000899	0.49	0.911	
Diet*Days	5	0.076817	0.076817	0.015363	8.41	0.000	***
Repl*Days(Diet)	12	0.013464	0.013464	0.001122	0.61	0.821	
Error	54	0.098697	0.098697	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	-0.031423	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(Diets)	12	1.348	0.119	0.010	0.15	1.000	
Diets*Days	5	15.758	15.777	3.155	48.13	0.000	***
Repl*Days(Diets)	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. Comparison 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	Signifi-cant
Constant		1.02143	0.01985	51.45	0.000	
Days		0.55467	0.00595	93.17	0.000	
Days*Diets						
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	0.01355	0.41	0.681	Ns
Control	0.716	0.16129	0.01336	12.07	0.000	***
EN/algae	0.623	0.06828	0.01309	5.22	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
Diet	5	270.933	8.073	1.615	10.84	0.000	
Day	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*Day	5	23.108	22.794	4.559	30.60	0.000	***
Repl*Day(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* fed on various diets (M1-PL stages).

Term	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
Constant		1.89668	0.05145	36.87	0.000	
Day		0.36485	0.006711	54.36	0.000	
Day*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	*
Control	0.548	0.18355	0.01503	12.22	0.000	***
EN/Algae	0.304	-0.06108	0.01501	-4.07	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 a mean ± 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

Experiment 2

Survival

In this experiment, the effect of pigmented and/or lipid-enriched nematodes and three different algal co-feed regimes together with the nematodes were investigated on larval growth, survival and larval development of *P. indicus*. Larval survivals on various feeds from PZ1 to PL stages are shown in Figures 3 and 5. Table 7 summarises mortality rates, final larval survivals and statistical comparisons of these data expressed in different superscripts. The feeding regimes used in the present experiment significantly ($P<0.001$) affected larval survival from PZ1 to PL stages (Tables 8a, b). When larvae were fed nema-

todes with an algal co-feed (EN/Alg1 and EN/Alg2) for a short period of time, they showed significantly lower mortality rates (3.43-4.99 % day^{-1}) compared to the control (6.78 % day^{-1}) and EN nematode fed treatments. PEN nematodes gave the lowest mortality rate (2.37 % day^{-1}) resulting in 68 % survival at metamorphosis. The EN nematodes which caused significantly the highest larval mortality rate (11.09 % day^{-1}) gave also the lowest final survival (10.33 %) at PL stages ($P<0.05$). NEN nematodes resulted in lower larval mortality rate (4.14 % day^{-1}) with a final survival of 51 % to PL stage. Figures 3, 5 and Table 7 demonstrate that all the nematode regimes, except EN nematodes, promoted significantly better larval survival rate and final survival than those of the control treatment.

Growth

(a) PZ1-PZ3/M1

Growth of larvae during protozoeal stages (day 1 - day 4) are presented in Figures 4a and 6a. Tables 9a and 9b show that there was significant effect of various nematode regimes on larval growth rate of *P. indicus* from PZ1 to PZ3/M1 stage. 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 (see Figure 4a).

Comparison of larval growth rates between day 1 and day 4 revealed that (Table 7) EN/Alg1 diet supported the highest larval growth rate (0.93 mm day^{-1}) compared to those obtained on all other diets. Nematode only fed larvae displayed significantly lower growth rates (0.54-0.57 mm day^{-1}) compared to those cultured on either algae alone (0.85 mm day^{-1}) or where algal co-feed were offered in conjunction with the nematodes (0.83-0.93 mm day^{-1}). PEN nematode fed larvae showed significantly better growth rate (0.57 mm day^{-1}) than those fed on either EN (0.54 mm day^{-1}) or NEN nematodes (0.54 mm day^{-1}).

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 survival and growth are means ± s.d. (n=3).

Diets	Mortality rate (% day^{-1})	Final Survival at PL (%)	Growth rate (mm day^{-1})		Final growth at PL (mm)
			PZ1-PZ3/M1	M1-PL	
NEN	4.142 ^{d}	51.00 ^{b} ± 5.07	0.535 ^{e}	0.358 ^{b}	5.48 ^{b} ± 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}	5.64 ^{b} ± 0.10
Control	6.784 ^{b}	32.00^{d} ± 2.60	0.851 ^{b}	0.599 ^{a}	7.49 ^{a} ± 0.18

Growth

(b) M1-PL

Figures 4b and 6b show that growth rate of the larvae fed various nematode feeds during mysis and PL stages were significantly lower (P<0.001) than larvae fed live mixed algae with five *Artemia* nauplii ml^{-1} (see Tables 10a, b). Table 7 shows that although the larvae fed nematodes and algal co-feeds had significantly higher growth rates during the herbivoral stages, these larvae displayed significantly lower growth rates (0.30-0.32 mm day^{-1}) compared to either the control diet (0.60 mm day^{-1}) or nematode diets alone (NEN and PEN nematode fed larvae) between M1 and PL stages. EN nematode fed larvae exhibited slower growth rate (0.28 mm day^{-1}) than that of (0.36 mm day^{-1}) NEN nematode fed larvae. Pigmented nematodes (PEN) gave significantly better growth rate (0.39 mm day^{-1}) than EN nematode fed ones between M1 and PL stages. Comparisons of final larval growths at PL stages (day 10) indicated that larvae reared on live mixed algae plus *Artemia* during mysis stages showed significantly (P<0.001) the greatest larval total length (7.49 mm TL). Apart from larvae fed EN nematodes, which had significantly the lowest growth (4.89 mm TL), all other diets promoted similar final total lengths (P>0.05) at PL stages (Table 7). Growth and survival during larval development of *P. indicus* larvae are shown in Tables 12a and 12b.

Figure 3. Survival (%) of *P. indicus* larvae reared on various feeding regimes from PZ1 to PL stages. Each value is a mean (n=3). (EN/Alg1= Lipid-enriched nematodes plus 30 cells μl^{-1} algae for 24 h at PZ1 stage, EN/Alg2= Lipid-enriched nematodes plus 30 cells μl^{-1} algae for 48 h at PZ1 stage, EN/Alg3 = Lipid-enriched nematodes plus 15 cells μl^{-1} algae for 48 h at PZ1 stage, Control= Mixed algae during protozoeal stages and plus *Artemia* during later stages).

Figures 4a, b. Growth (mm) of *P. indicus* larvae reared on various feeding regimes (a) from PZ1 to PZ3/M1 and (b) from M1 to PL stages. Each value is a mean (n=3). (EN/Alg1= Lipid-enriched nematodes plus 30 cells μl^{-1} algae for 24 h at PZ1 stage, EN/Alg2= Lipid-enriched nematodes plus 30 cells μl^{-1} algae for 48 h at PZ1 stage, EN/Alg3= Lipid-enriched nematodes plus 15 cells μl^{-1} algae for 48 h at PZ1 stage, Control= Mixed algae during protozoeal stages and plus *Artemia* during later stages).

Figure 3

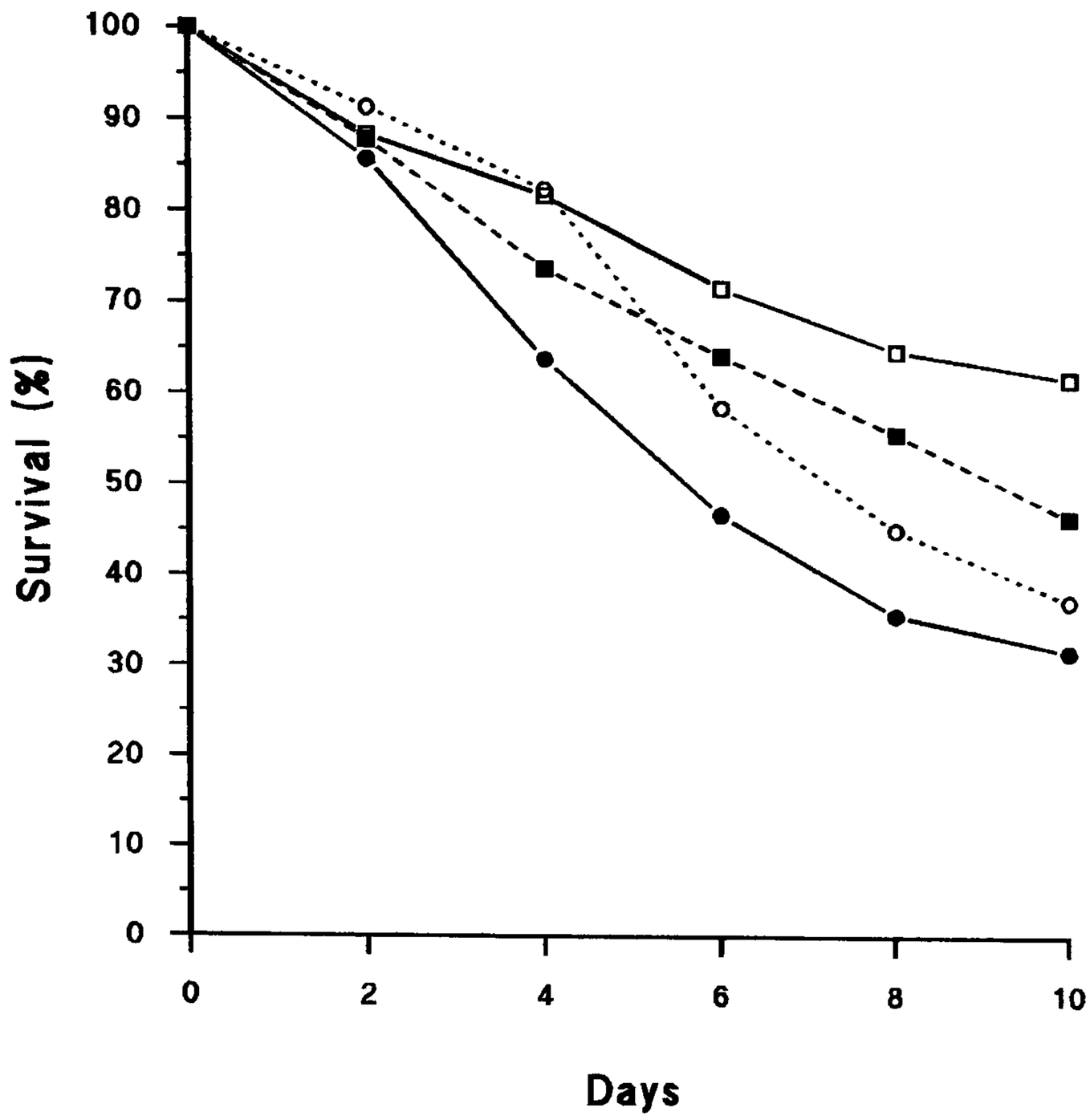


Figure 4a

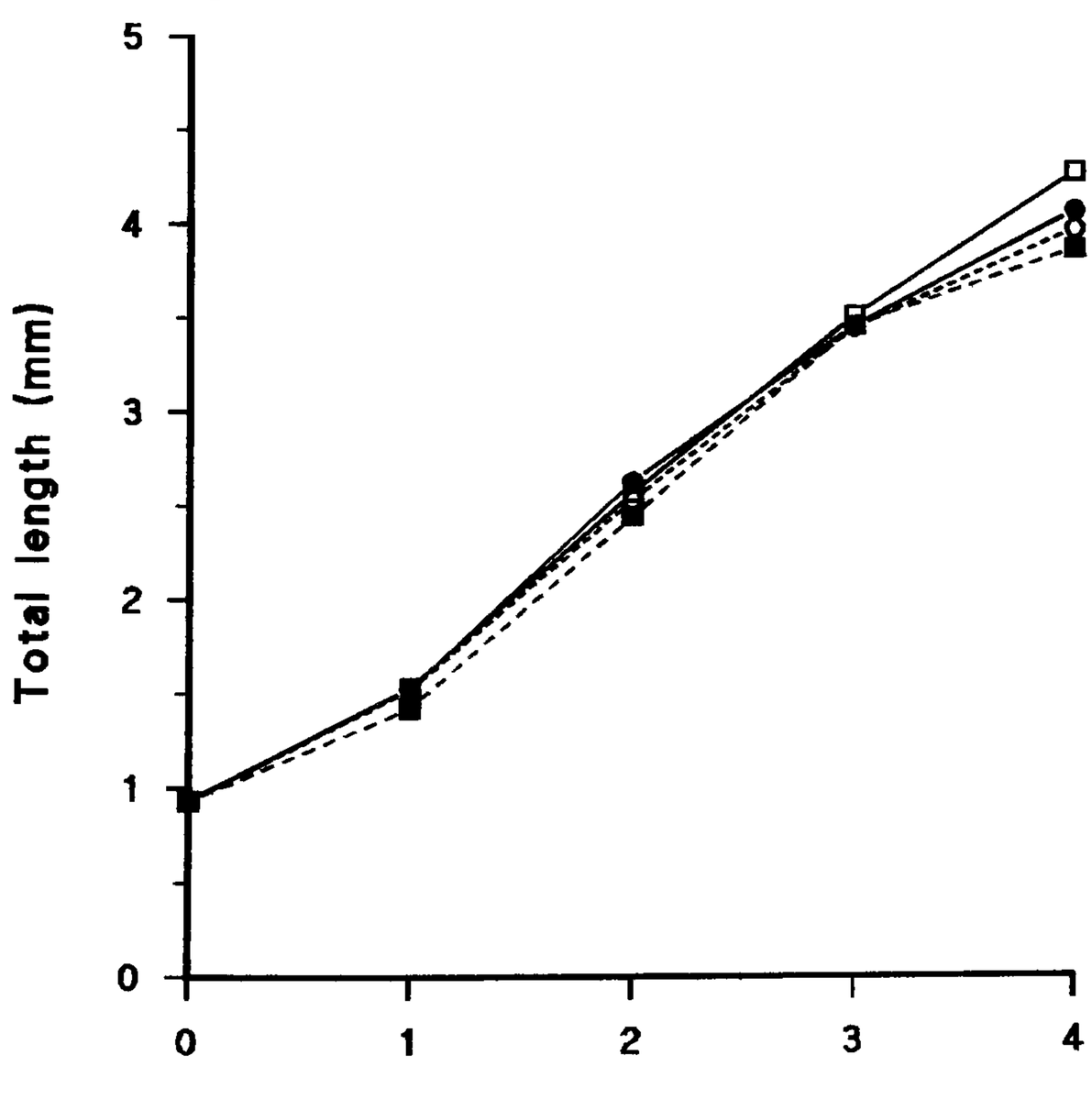


Figure 4b

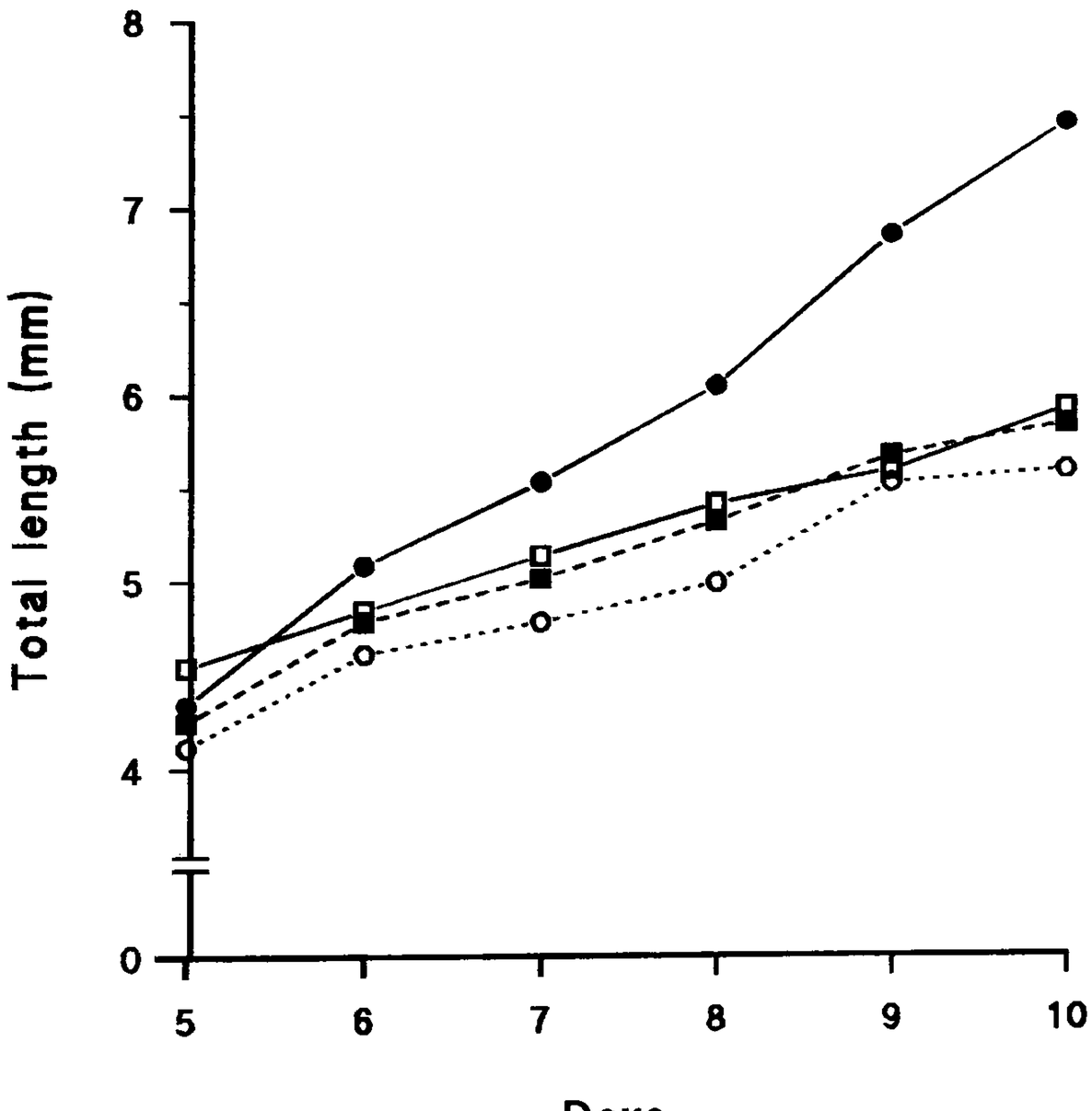


Figure 5. Survival (%) of *P. indicus* larvae reared on various feeding regimes from PZ1 to PL stages. Each value is a mean (n=3). (EN= Lipid-enriched, NEN= Non-enriched, PEN= Pigmented and lipid-enriched, Control= Mixed algae during protozoeal stages and plus *Artemia* during later stages).

Figures 6a, b. Growth (mm) of *P. indicus* larvae reared on various feeding regimes (a) from PZ1 to PZ3/M1 and (b) from M1 to PL stages. Each value is a mean (n=3). (EN= Lipid-enriched, NEN= Non-enriched, PEN= Pigmented and lipid-enriched, Control= Mixed algae during protozoeal stages and plus *Artemia* during later stages).

Figure 5

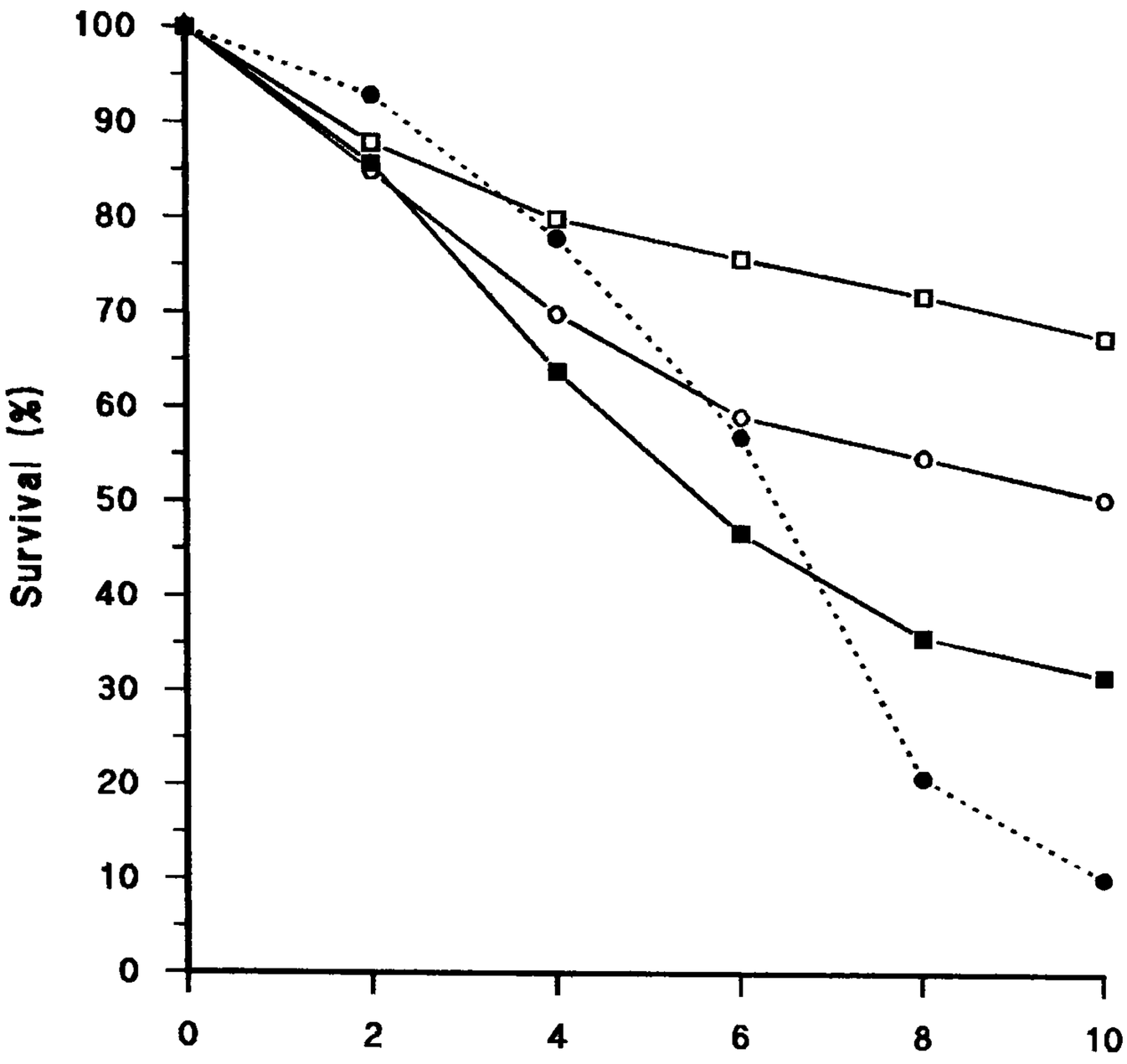


Figure 6a

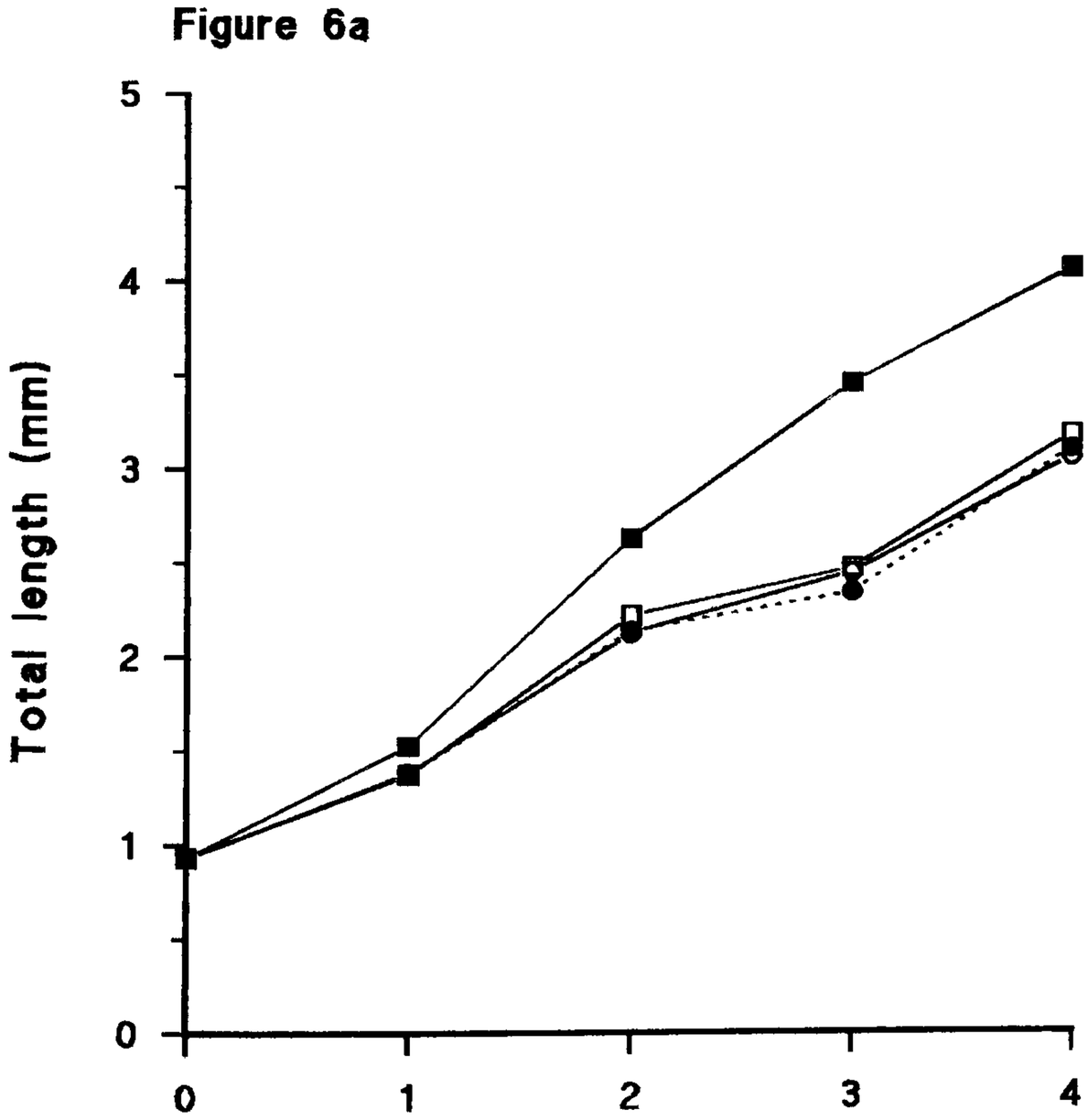
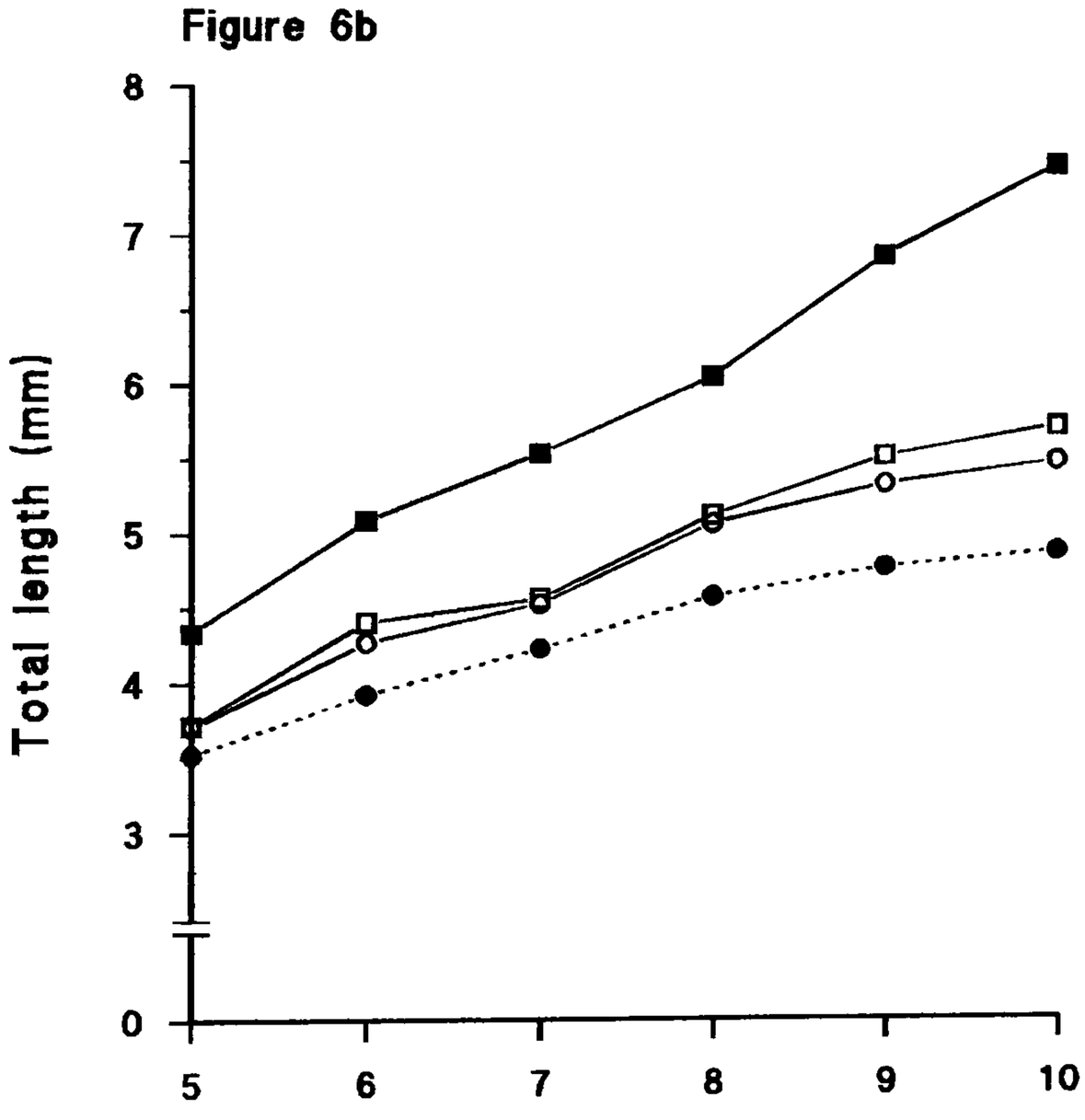


Figure 6b



● EN ■ Control

○ NEN □ PEN

Larval development

On day 4, larvae reared on all single nematode feeds (NEN, EN and PEN) were at PZ3 stage whereas more than 50 % of the larvae fed either nematodes/algal co-feeds or algae alone (control) were at M1 stage (see Table 11). Control diet fed larvae were at stage PL4 (73 %) on day 10 while EN nematodes fed larvae were still between M2 and PL1 stages. Development of larvae fed PEN nematodes or nematodes/algal co-feeds were slightly faster than EN and NEN nematode fed larvae (at PL1/PL2). Larvae fed the control diet, EN/Alg1-3 and PEN started to metamorphose into PL1 stage on day 7, 8 and 9 respectively. EN nematode fed larvae started to reach this stage on day 10.

Trypsin activity

Trypsin activities (per larva and per µg) of the larvae reared on the nematode, mixed live algae, and the nematode plus 15 cells μl^{-1} algal co-feed for 48 h were determined in this trial. Figure 7 shows that the algal co-feed along with EN nematodes induced significantly ($P>0.001$) higher total and specific trypsin activities (33.36×10^{-4} IU $larva^{-1}$ and 4.81×10^{-4} IU μg^{-1} dry weight = DW) than that of larvae fed nematodes only (18.44×10^{-4} IU $larva^{-1}$ and 2.85×10^{-4} IU μg^{-1} DW). Mixed algae fed larvae, however, displayed the highest trypsin activities (47.53×10^{-4} IU $larva^{-1}$ and 6.64×10^{-4} IU μg^{-1} DW).

Nutritional composition of the nematode feeds

Composition (% dry weight) of protein, lipid and fatty acid profile of the nematodes used in the present experiment are shown in Table 13. NEN nematodes showed high level of protein (66 %) compared to EN (52.3 %) and PEN (44.2 %) nematodes. Lipid-enrichment remarkably increased lipid content of the PEN nematodes (32.90 %) in comparison to NEN nematodes. Yet, lipid content of the EN nematodes was even lower (9.6 %) than that of the NEN nematodes (14.1 %). Lipid-enriched nematodes (EN and PEN) contained substantially higher ω-3 PUFA (3-5 times) particularly 20:5ω-3

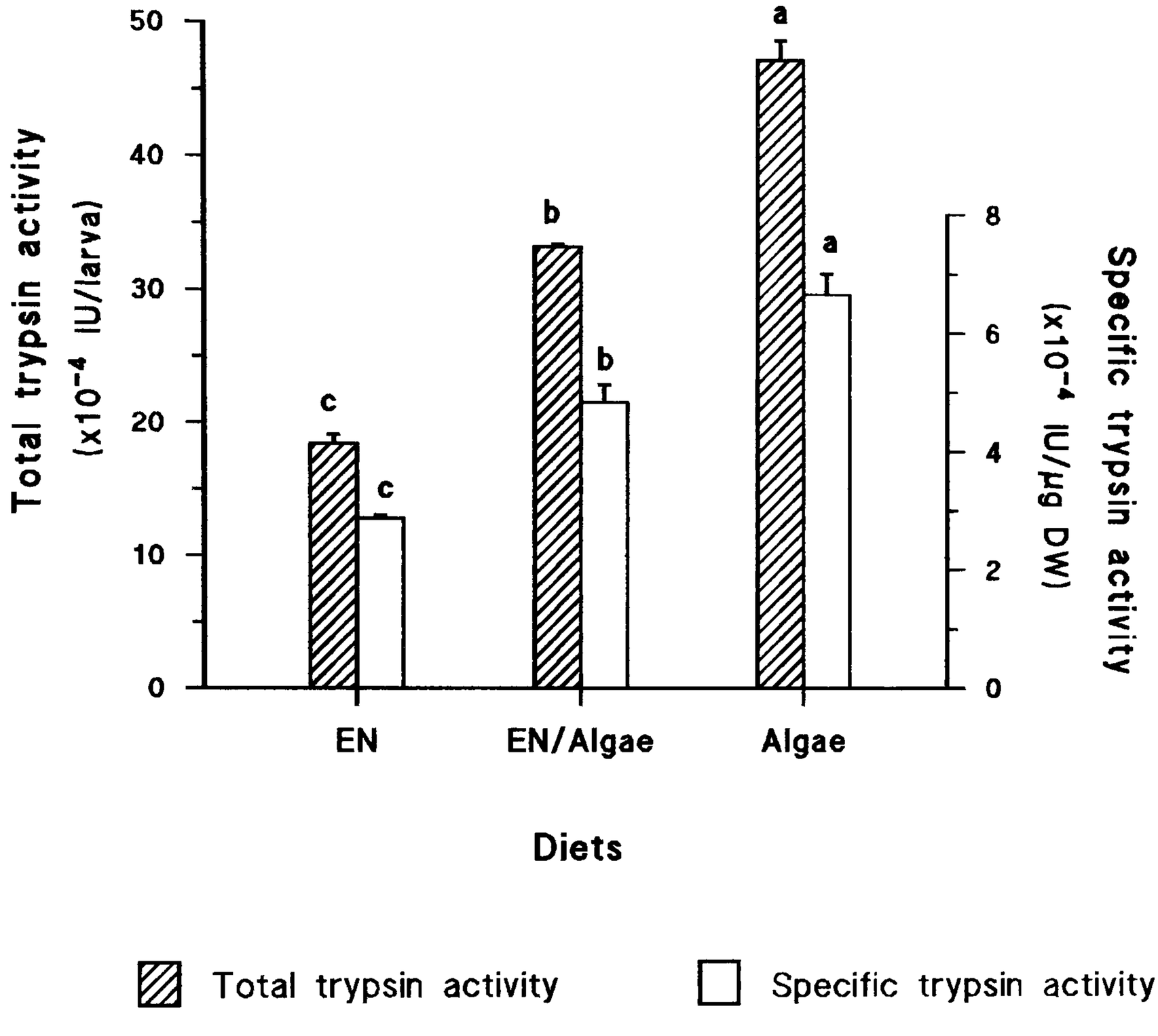


Figure 7. Total and specific trypsin activity of larvae reared on lipid–enriched nematodes (EN), EN with algae as a co–feed (15 cells/µl) and algae from PZ1 to PZ2 stages. Each bar is a mean ± s.d. (n=2).

(eicosapentaenoic acid) (5.85-10.99 %) compared to NEN nematodes (1.97 %). In spite of the lack of docosahexaenoic acid (22:6ω-3) in NEN nematodes, lipid enriched nematodes had 1.65 % (EN) and 4.48 % (PEN) of this fatty acid. In general, the NEN nematodes were richer in ω-6 fatty acids whereas EN and PEN nematodes were richer in ω-3 fatty acids. Ratio of ω6/ω3 of the EN nematodes were 2.20-2.61 % whereas this ratio was 20.05 % in the NEN nematodes (see Table 13).

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 SS	Adj MS	F	P	Significant
Diet	6	8190.5	1903.8	317.3	10.68	0.000	
Day	1	27497.2	27497.2	27497.2	925.48	0.000	
Repl(Diet)	14	238.0	217.1	15.5	0.52	0.911	
Diet*Day	6	6215.5	6215.5	1035.9	34.87	0.000	***
Repl*Day(Diet)	14	295.8	295.8	21.1	0.71	0.755	
Error	63	1871.8	1871.8	29.7			
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 SS	Adj MS	F	P	Significant
Diet	6	88.214	1.086	0.181	4.33	0.000	
Day	1	581.776	578.248	578.248	1.4E+04	0.000	
Repl(Diet)	14	0.639	0.286	0.020	0.49	0.940	
Diet*Day	6	26.953	27.052	4.509	107.82	0.000	***
Repl*Day(Diet)	14	0.668	0.668	0.048	1.14	0.317	
Error	858	35.880	35.880	0.042			
Total	899	734.130					

Table 9b. Comparison 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	Signifi-cant
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	0.01501	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 SS	Adj MS	F	P	Signifi-cant
Diets	6	256.039	22.705	3.784	22.50	0.000	
Days	1	503.425	516.268	516.268	3069.17	0.000	
Repli(Days)	14	4.755	1.810	0.129	0.77	0.704	
Diets*Days	6	37.232	37.602	6.267	37.26	0.000	***
Repli*Days(Diets)	14	2.464	2.464	0.176	1.05	0.404	
Error	1329	223.552	223.552	0.168			
Total	1370	1027.466					

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

Term	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
Constant		3.74380	0.02518	148.68	0.000	
Day		0.362321	0.00654	55.40	0.000	
Day*Diets						
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 (%) (Day 4)	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 5. 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	78.00± 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	45.50± 2.65	72.33± 2.75	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	1.52±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	2.54±0.03	2.22±0.01	2.63±0.05
3	2.46±0.01	2.35±0.04	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. Percentage nutritional and fatty acid composition of the nematode diets used in the present experiment to feed *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
Monounsaturates (%)	23.87	23.88	35.76
Polyunsaturates (%)	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

Experiment 3

Survival

(a) PZ1-PZ3/M1

Figure 8a shows survival of *P. indicus* larvae on various feeding regimes between PZ1 and PZ3/M1 stages. Statistical analyses for protozoeal stages showed significant differences between the effect of diets on larval survival during these stages ($P<0.001$) (see Tables 16a, b). Highest mortality rates were found in the larvae fed NEN nematodes (7.55 % day^{-1}) and the control diet (6.56 % day^{-1}). CLO lipid-enriched nematodes gave significantly lower mortality rate (2.3 % day^{-1}) compared to EN (3.97 % day^{-1}) and MAR (5.40 % day^{-1}) (Table 14). Comparisons of larval survival of M1 larvae showed that all the lipid enriched nematodes had significantly ($P<0.01$) higher survival (73 -88 %) than either larvae fed on NEN nematodes (61 %) or those fed live algae (68 %) (see Figure 8a and Table 15). Although CLO nematodes promoted the highest survival until M1 stage (88 %), there was no significant difference ($P>0.05$) between the various lipid-enriched treatments between PZ1 and M1 stage.

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}

(b) M1-PL1

Figure 8b displays survival of the larvae fed various diets between M1 and PL stages. Comparison of the larval growth rates (Tables 17a, b) demonstrated no significant effects of various feeding regimes on larval growth during

Figures 8a, b. Survival (%) of *P. indicus* larvae reared on various feeding regimes from PZ1 to PZ3/M1 and from M1 to PL stages. Each value is a mean (n=3). (MAR= Marella 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).

Figures 9a, b. Growth (mm) of *P. indicus* larvae reared on various feeding regimes from PZ1 to PZ3/M1 and from M1 to PL stages. Each value is a mean (n=3). (MAR= Marella 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).

Figure 8a

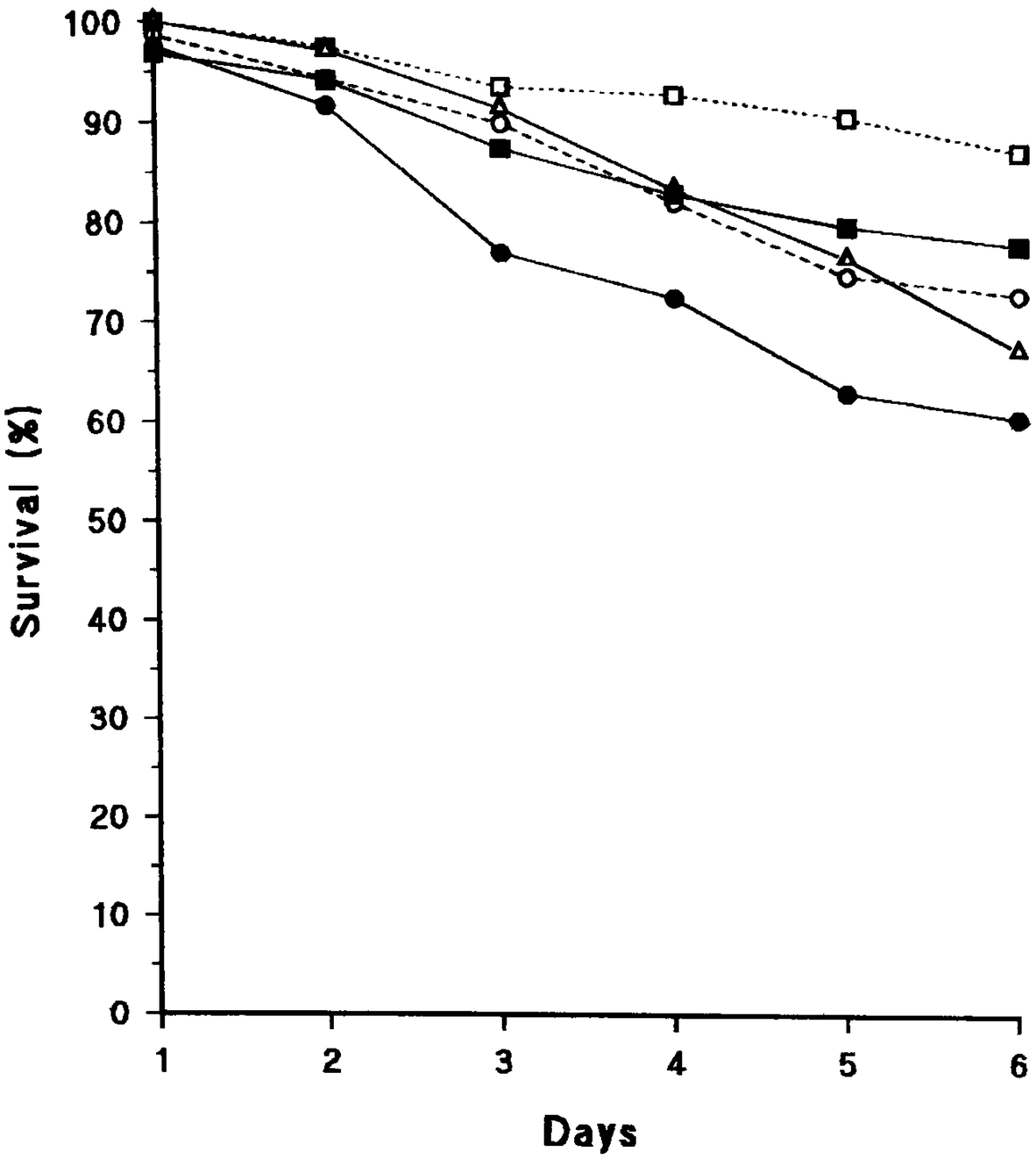


Figure 8b

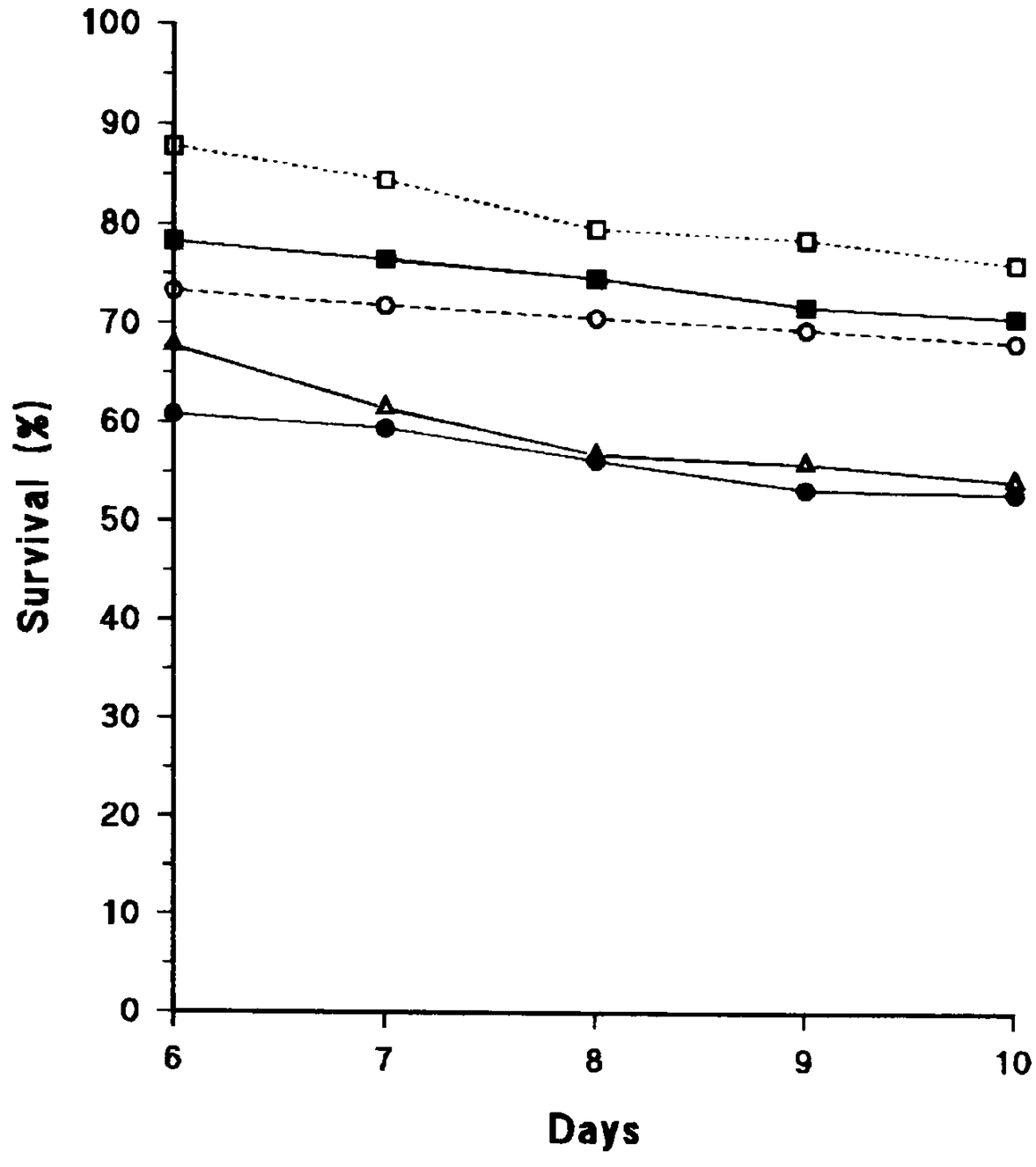


Figure 9a

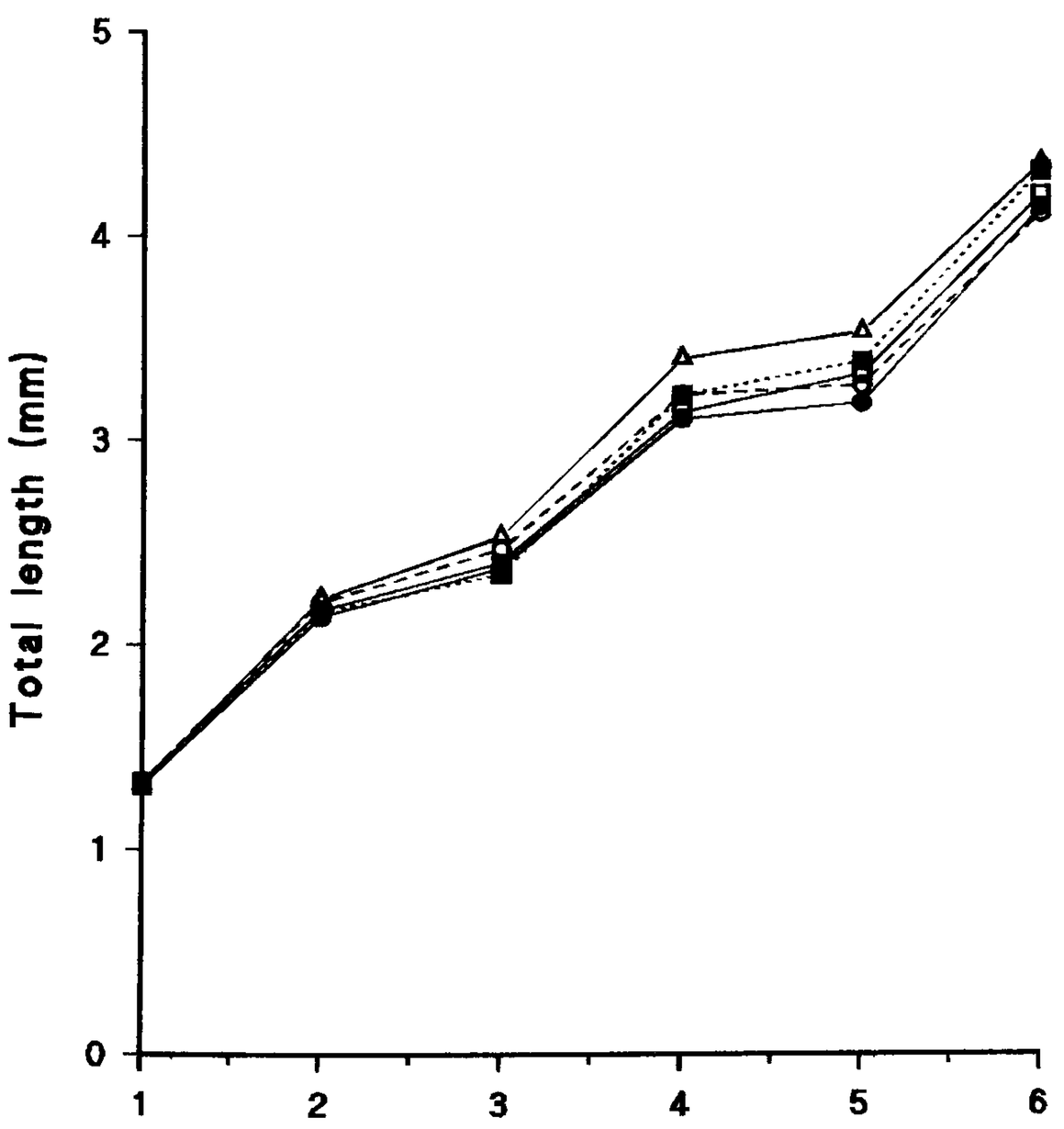
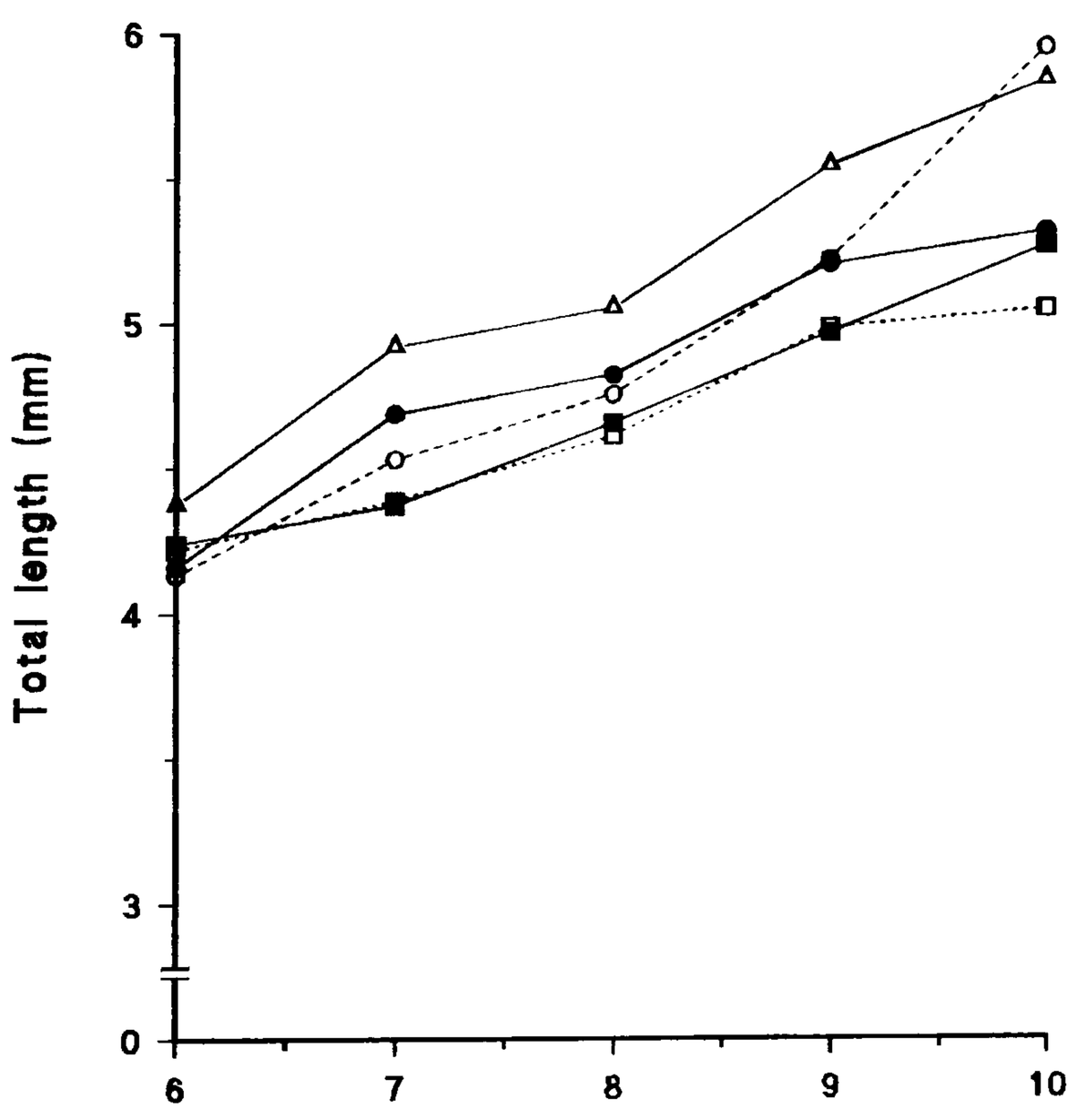


Figure 9b



mysis stages until PL1 (P>0.05). In comparison to protozoeal stages, Figure 10b clearly shows that larval mortality declined during mysis stages. Comparison of survivals at PL1 stage also showed that NEN nematodes and control diet resulted in significantly lower survivals compared to other nematode feeding regimes (P<0.05). Survivals at metamorphosis on CLO, MAR, and EN nematodes were 77 %, 69 %, 72 % respectively. Table 15 shows that there was no significant difference between survivals of larvae fed various lipid-enriched nematodes (P>0.05). All the survival data for Figures 8a and 8b are shown in Table 20a.

Table 15. 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).

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^{b} ±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

Growth

(a) PZ1-PZ3/M1

Growth rates of larvae fed various feeds during protozoeal stages (Figure 9a) were significantly (P<0.05) different (Tables 18a, b). Highest growth rates were achieved with the EN nematodes (0.574 mm day^{-1}) and the control diet (0.576 mm day^{-1}) (Table 14). Larvae fed CLO nematodes showed superior growth (0.536 mm day^{-1}) to larvae reared on either MAR (0.516 mm day^{-1}) or NEN nematodes (0.523 mm day^{-1}). Growth rates of larvae fed MAR and NEN nematodes were not significantly different. Comparison of larval growth rates at M1 stage using one-way ANOVA demonstrated that EN nematodes and the mixed live algae promoted greatest (P<0.05) total lengths (4.37 and 4.38

mm TL) compared with the other nematode feeds (see Table 15). Total lengths of larvae fed NEN, MAR, and CLO nematodes ranged from 4.18 mm to 4.23 mm ($P>0.05$).

(b) M1-PL1

Figure 9b shows growth of larvae during mysis and early postlarval stages. Comparison of larval growth rates between M1 and PL stages (Tables 19a, b) showed that MAR nematodes gave the highest growth rate (0.425 mm day^{-1}) compared to all other lipid-enriched nematodes and even the control diet (0.360 mm day^{-1}). 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}). Table 15 shows that there was a significant difference in total length of PL1 larvae fed on different diets ($P<0.001$). Control treatment (algae until M2 and *Artemia* from M1 to PL1) and MAR nematode fed larvae supported the highest mean total lengths (5.71 mm and 5.66 mm respectively). Although CLO nematodes showed greater TL (5.37 mm) at PL1 stage than those fed NEN nematodes (5.28 mm), the difference was not significant ($P<0.05$). Growth data for Figures 9a and 9b are summarised in Table 20b.

Larval development

Larval development (days) from PZ1 to M1 and PL1 stages were determined when the larvae started to developed into these particular stages. Table 15 shows that CLO, and EN nematode fed larvae, as well as the control developed into M1 stage one day earlier than larvae fed MAR and NEN nematodes. Larvae fed the lipid-enriched nematodes metamorphosed into PL1 stage one day earlier than those fed NEN nematodes. Yet, larval duration until metamorphosis was shortest (8 days) in larvae fed the control treatment.

Nutritional composition of the nematode feeds

Tables 21a and 21b show the nutritional composition and fatty acid profile of the nematodes used during the first and second week of the present study.

Protein content of NEN nematodes (74.4-77 %) was higher than EN (52.9-64.6 %), MAR (55.4-59,1 %) and CLO (51.9-56.7 %) enriched nematodes. All lipid-enriched nematodes had slightly higher lipid content in comparison to NEN nematodes (see Tables 21a, b). The lipid-enriched nematodes contained substantially higher levels of ω-3 PUFA (10.62-14.9 %) than NEN nematodes (2.21-2.94 %). Among the ω-3 series, lipid-enriched nematodes were also richer in 20: 5ω-3 fatty acids (5.17-6.83 %) than NEN (1.29-1.42 %). Similarly, 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 HUFA level of ω-6 series in lipid-enriched nematodes were comparable to that in NEN nematodes. In general, fatty acid profiles of the lipid-enriched nematodes, irrespective of the lipid source, were similar. The ratios of ω-6/ω-3 HUFA of lipid enriched nematodes ranged from 1.87 to 3.16 % compared to NEN nematodes (13.49-17.07 %). Tables 21a and 21b show that the nutritional content and fatty acid profile of the nematodes (enriched or non-enriched) used to feed *P. indicus* during larval development did not differ greatly.

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	Significant
Diets	4	2369.68	147.55	36.89	3.55	0.012	
Days	1	6804.30	6644.12	6644.12	639.35	0.000	
Repl(Diets)	10	280.35	132.47	13.25	1.27	0.266	
Days*Diets	4	918.59	911.35	227.84	21.92	0.000	***
Repl*Days(Diets)	10	147.90	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 stages.

Term	Mortality rate (% day^{-1})	Coeff.	Stdev.	t-value	P	Significant
Constant		104.117	0.802	129.79	0.000	
Days		-5.1552	0.2039	-25.29	0.000	
Days*Diets						
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	Signifi-cant
Diets	4	6397.36	193.63	48.41	2.74	0.040	
Days	1	685.91	697.22	697.22	39.53	0.000	
Replic(Diets)	10	361.43	289.51	28.95	1.64	0.126	
Diets*Days	4	125.88	66.79	16.70	0.95	0.446	Ns
Replic*Days(Diets)	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. Comparison of larval mortality rates (% day^{-1}) of *P. indicus* fed various feeding regimes between M1 and PL stages.

Term	Mortality rate (% day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
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	Signifi-cant
Diets	4	3.121	0.576	0.144	3.38	0.009	
Days	1	847.713	847.347	847.347	2.0E+04	0.000	
Replic(Diets)	10	0.203	0.057	0.006	0.13	0.999	
Diets*Days	4	1.851	1.846	0.462	10.85	0.000	***
Replic*Days(Diets)	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 regimes from PZ1 to PZ3/M1 stages.

Term	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Signifi-cant
Constant		0.88626	0.01533	57.80	0.000	
Days		0.545019	0.003861	141.15	0.000	
Days*Diets						
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	-0.008760	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	Signifi-cant
Diets	4	31.2475	5.3624	1.3406	15.32	0.000	
Days	1	170.9386	168.9534	168.9534	1930.75	0.000	
Replic(Diets)	10	2.2207	1.3715	0.1371	1.57	0.111	
Diets*days	4	10.2721	10.2672	2.5668	29.33	0.000	***
Replic*Days(Diets)	10	2.8645	2.8645	0.2865	3.27	0.000	
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	Signifi-cant
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	80.17±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	71.50±3.91	54.83±4.25

Table 20b. Data for Figures 9a and 9b. 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. Percentage nutritional and fatty acid composition of the nematode diets used in the first week of the present experiment to feed *P. indicus* larvae from PZ1 to PL stages. NEN= non-enriched, EN= capelin lipid-enriched, MAR= marilla lipid-enriched and CLO= cod liver lipid-enriched nematodes.

	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	2.98	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	29.4	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
Monounsaturates (%)	27.89	26.76	33.15	31.15
Polyunsaturates (%)	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. Percentage nutritional and fatty acid composition of the nematode diets used in the second week of the present experiment to feed *P. indicus* larvae from PZ1 to PL stages. NEN= non-enriched, EN= capelin lipid-enriched, MAR= marilla lipid-enriched and CLO= cod liver lipid-enriched nematodes.

	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
Monounsaturates (%)	24.76	33.59	23.19	30.43
Polyunsaturates (%)	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

Experiment 4

Survival

(a) PZ1-PZ3/M1

Figure 10a shows survival of *P. indicus* larvae fed various feeding regimes during protozoeal stages. Larval mortality rates were compared between day 3 and day 6 (Tables 24a, b) and results are summarised in Table 22. Lowest mortality rate (2.83 % day^{-1}) was achieved with larvae fed PEN nematodes whereas the highest mortality rate (17.68 % day^{-1}) was found in the larvae fed live mixed algae during protozoeal stages (see Figure 10a and Table 22). PLC and CLO nematodes fed larvae showed significantly higher mortality rates (4.22-6.30 % day^{-1}) than PEN nematode fed ones ($P<0.01$). Comparison of larval total lengths at M1 stage (Table 23) revealed that survival (91 %) of larvae at M1 stage on PEN nematodes was not significantly ($P>0.05$) different than those fed either CLO (88 %) or PLC nematodes (80 %). Control treatment resulted in only 33 % survival from PZ1 to M1 stage.

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 ($P>0.05$).

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}

(b) M1-PL1

Since survival data of the control larvae did not show a linear relationship, it was excluded in the calculations (Figure 10b). Comparisons of mortality rates between M1 and PL stages (Tables 25a, b) showed that neither of the diets had significantly different effects from each other on larval survival ($P>0.05$).

All the nematode feeds resulted in low mortality rates of between 0.72 and 3.27 % day^{-1} (see Table 23). There was also no significant difference in the final survival of larvae fed the three types of nematodes from PZ1 to PL1 stage (Table 23). Larvae (PZ1) fed PEN, CLO and PLC nematodes metamorphosed into PL1 stage with 88%, 79% and 78% survivals respectively. Only 11% of the larvae fed the control feed developed into PL1 stage. Survival data for Figures 10a, b is shown in Table 28a.

Table 23. 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).

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

(a) PZ1-PZ3/M1

Figure 11a shows growth of the larvae fed various feeding regimes from PZ1 to PZ3/M1 stages. Growth rates of larvae fed the nematode feeds and the control diet (Tables 26a, b) were significantly ($P<0.001$) different between PZ1 and PZ3/M1 (day 1-day 6). The control diet supported the highest larval growth rate (0.589 mm day^{-1}) during this period (see Table 22). PEN nematodes gave higher growth rate (0.455 mm day^{-1}) than PLC (0.415 mm day^{-1}) and CLO (0.440 mm day^{-1}) nematodes fed larvae. Comparisons of larval total lengths at M1 stage (Table 23) also indicated the same results in that PEN nematode fed larvae had significantly ($P<0.05$) greater lengths (3.88 mm TL) compared to those fed either PLC (3.77 mm TL) or CLO (3.79 mm TL)

nematodes. Live mixed algae, however, promoted significantly the greatest total length (3.92 mm TL) at M1 stage.

(b) M1-PL1

Figure 11b shows growth of the larvae between M1 and PL stages. Growth rates of larvae were significantly ($P<0.01$) affected by feeds during mysis and early PL stages (Tables 27a, b). Highest larval growth rate was obtained from those fed CLO nematodes (0.559 mm day^{-1}) between M1 and PL stages (see Table 22). Larval growth rates on PEN and PLC did not differ significantly during this period ($P>0.05$). Larvae fed the control diet showed the lowest growth rate (0.465 mm day^{-1}). Table 23 shows that final larval total length (on day 10) on PEN nematodes (5.18 mm) was significantly ($P<0.05$) greater than the larvae fed PLC diet (5.03 mm). Length of larvae fed the control diet was 5.23 mm at PL1 stage (Figure 11b). Growth data for Figures 11a, b are shown in Table 28b.

Larval development

Table 23 shows duration (days) of larval development to M1 and PL1 stages. PEN and CLO nematode fed larvae started to develop into M1 stage one day earlier (day 6) than those fed PLC nematodes (day 7). Larvae fed live mixed algae reached M1 stage on day 5, but more than 50 % of M1 larvae were observed on day 6. Larvae fed PEN and CLO again metamorphosed into PL1 stage on day 9 while PLC nematode fed larvae reached this stage on day 10. Metamorphosis occurred 1-2 days earlier in the control diet fed larvae.

Nutritional composition of the nematode feeds

Tables 29a, b show protein, lipid content and fatty acid profile of the nematodes used to feed *P. indicus* larvae in the first and second week of the culture. Protein levels of PLC, PEN and CLO were 55.6-59.6, 51.8-54.9 and 53.9-54 % respectively. Lipid contents of the PLC, PEN and CLO enriched nematodes were 34.6-35.2 %, 22.8-32 % and 31.9-38.5 % respectively. With

Figures 10a, b. Survival (%) of *P. indicus* larvae reared on various feeding regimes from PZ1 to PZ3/M1 and from M1 to PL stages. Each value is a mean (n=3). (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).

Figures 11a, b. Growth (mm) of *P. indicus* larvae reared on various feeding regimes from PZ1 to PZ3/M1 and from M1 to PL. Each value is a mean (n=3). (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).

Figure 10a

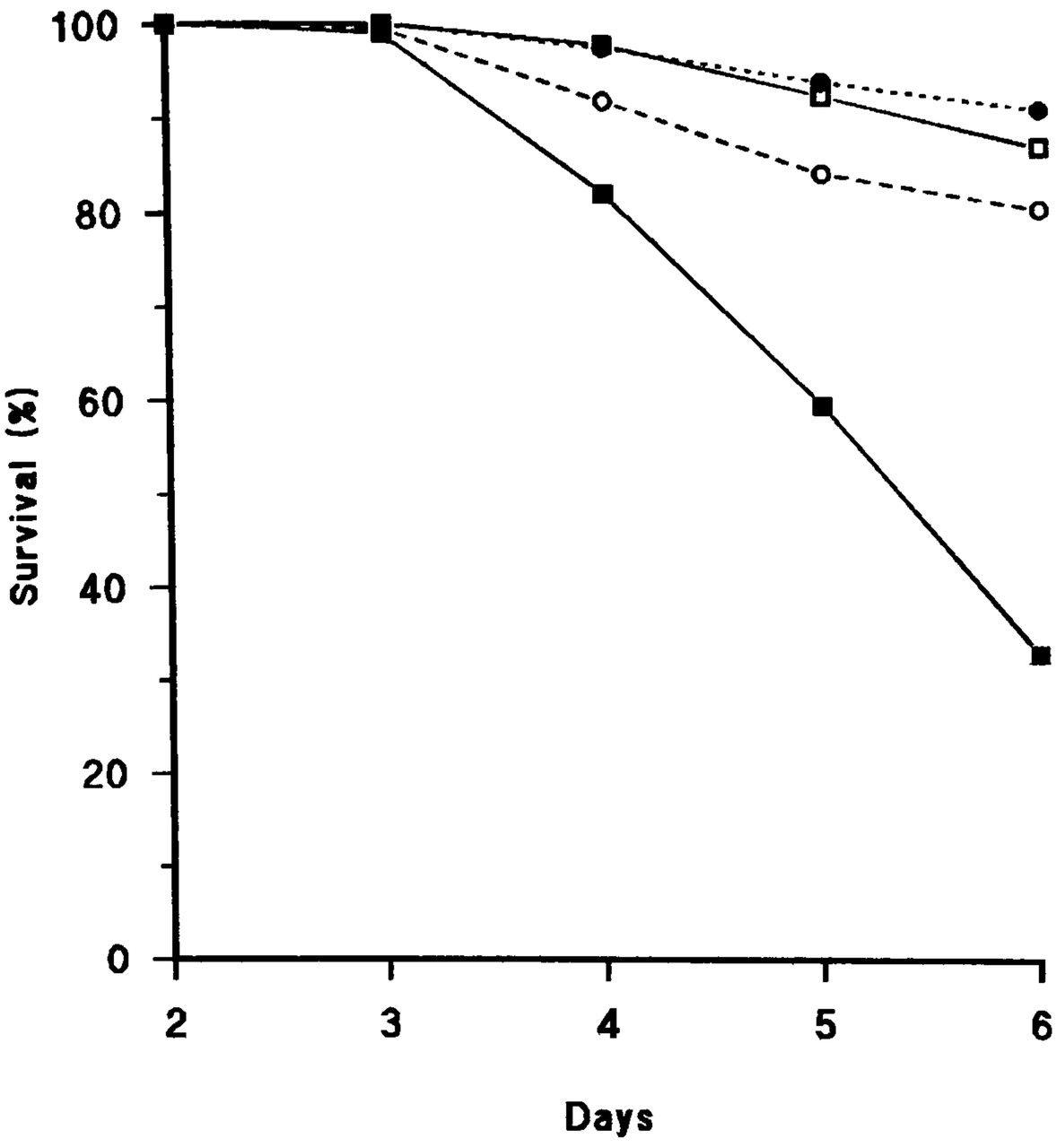


Figure 10b

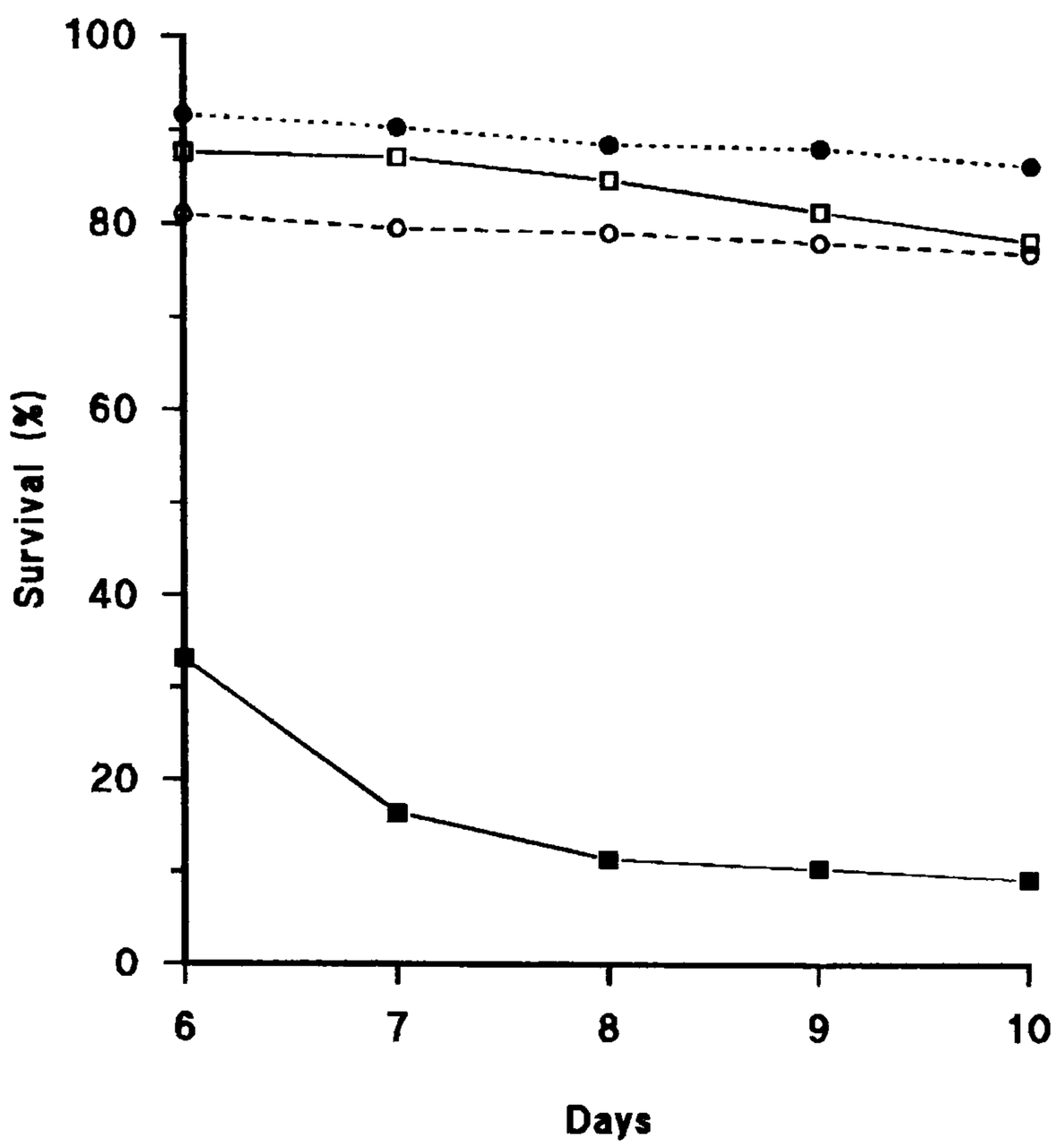


Figure 11a

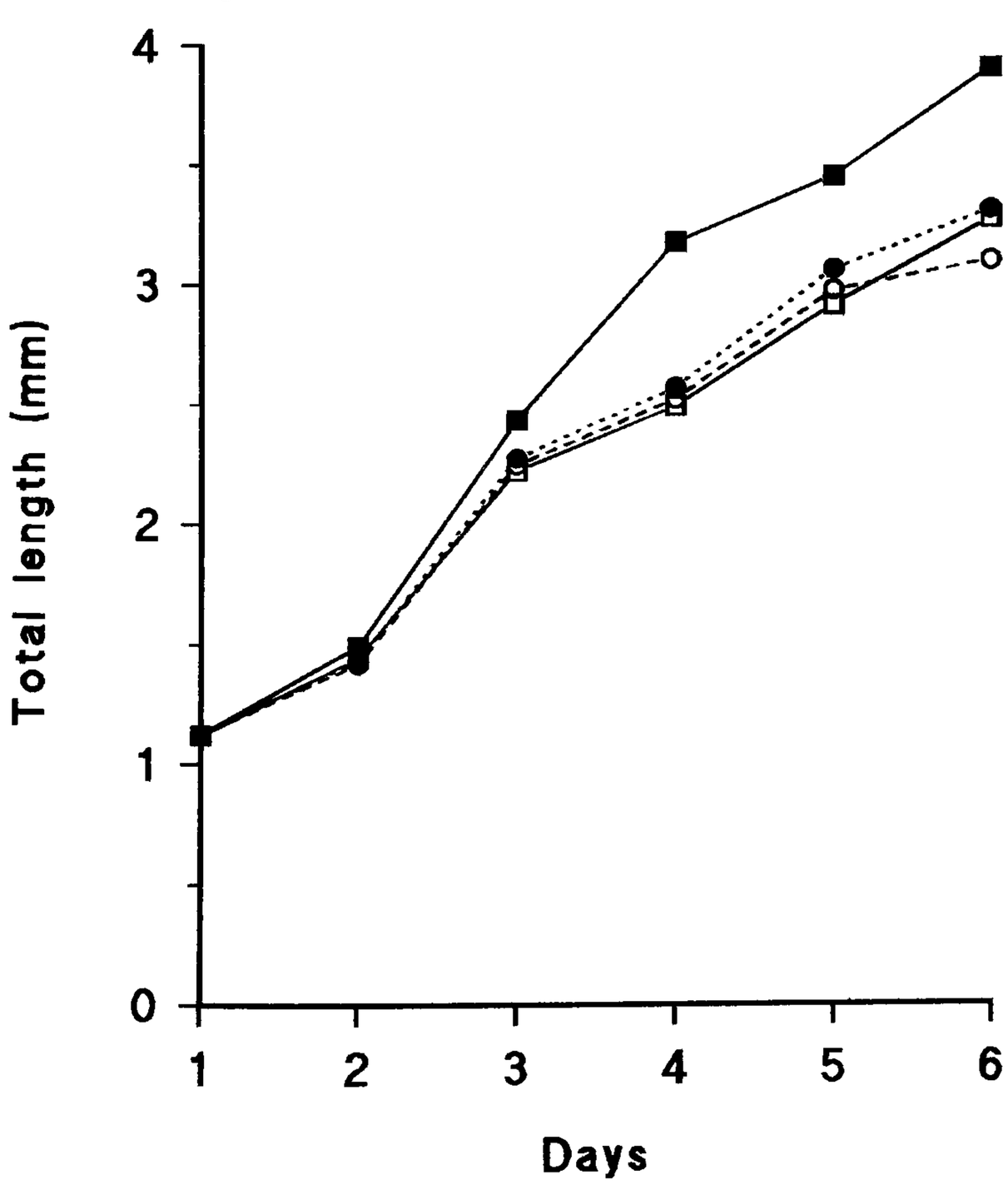
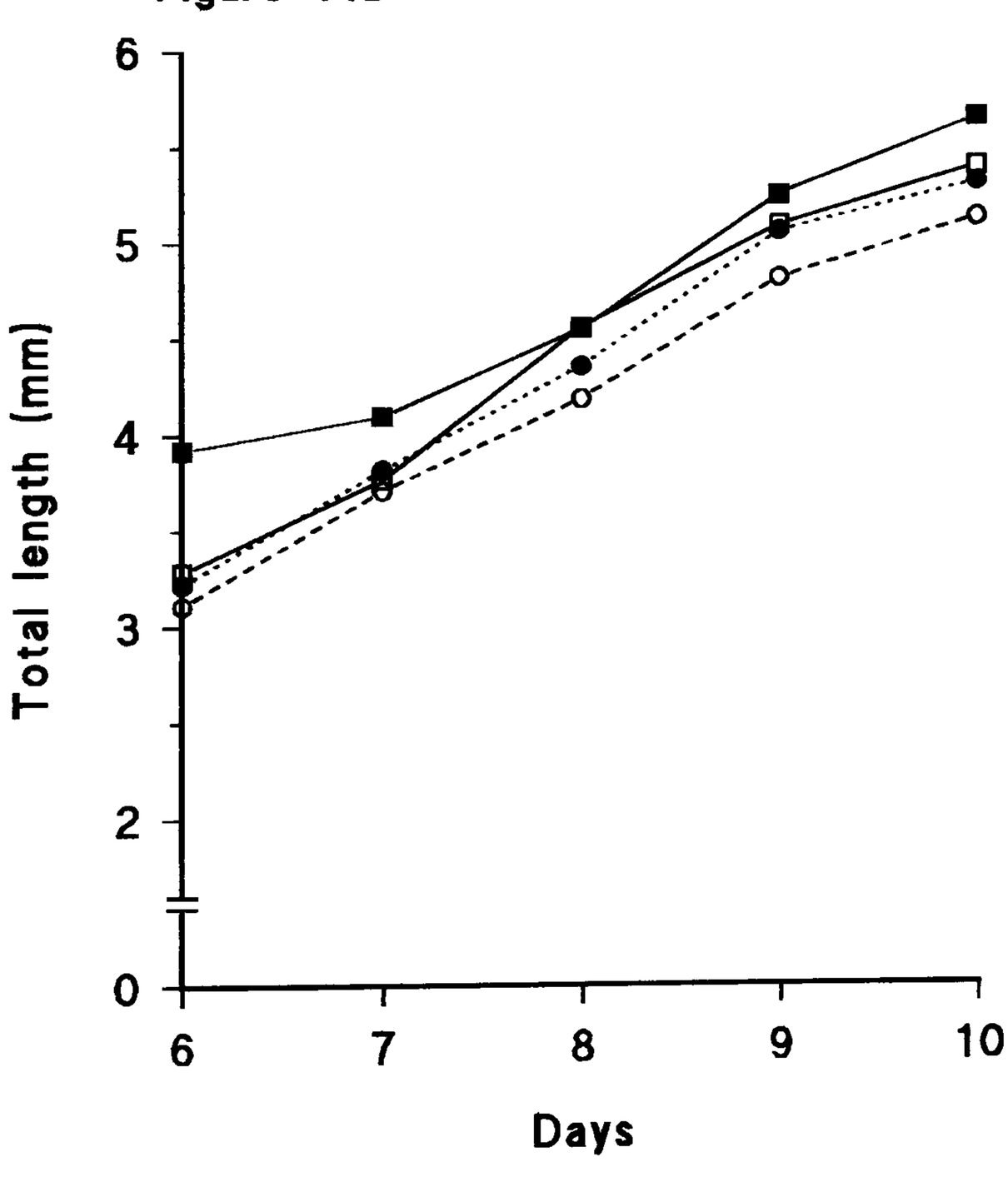


Figure 11b



● PEN ○ PLC □ CLO ■ Control

regard to PUFA, ω-3 types of fatty acid contents of the PLC nematodes were slightly lower (10.37-12.44 %) in comparison to PEN (14.7-15.28 %) and CLO nematodes (13.29-17.39 %). Yet, ω-6 series of the 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 %). Eicosapentaenoic acid (20: 5ω-3) content of the PLC (4.49-7.96 %), PEN (7.59-9.01) and CLO (6.04-9.22 %) were all comparable. Docosahexaenoic acid (22:6ω-3) content of all the nematode feeds (PLC = 2.57-3.22, PEN = 4.02-4.1 and CLO = 4.24-5.24) were also similar.

Table 24a. Two-way ANOVA with day as a covariate on survival of *P. indicus* larvae from PZ2 to PZ3/M1 (day 3 to day 6) on various feeding regimes.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	3	5819.27	553.67	184.56	16.29	0.000	
Days	1	4686.08	4686.08	4686.08	413.67	0.000	
Repl(Diets)	8	253.13	12.33	1.54	0.14	0.997	
Diets*Days	3	3556.39	3556.39	1185.46	104.65	0.000	***
Repl*Days(Diets)	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	Significant
Diets	2	770.311	79.326	39.663	4.37	0.023	
Days	1	158.669	158.669	158.669	17.46	0.000	
Repl(Diets)	6	367.200	166.931	27.822	3.06	0.020	
Diets*Days	2	38.406	38.406	19.203	2.11	0.140	Ns
Repl*Days(Diets)	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	Signifi-cant
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	Signifi-cant
Diets	3	21.719	0.130	0.043	0.92	0.430	
Days	1	308.786	308.893	308.893	6554.06	0.000	
Repl(Diets)	8	0.790	0.305	0.038	0.81	0.595	
Diets*Days	3	6.094	6.122	2.041	43.30	0.000	***
Repl*Days(Diets)	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	Signifi-cant
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 from M1 to PL stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Signifi-cant
Diet	3	14.017	3.376	1.125	10.85	0.000	
Days	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*Days	3	1.376	1.444	0.481	4.64	0.003	**
Repl*Days(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	Signifi-cant
Constant		0.80952	0.06228	13.00	0.000	
Days		0.517013	0.008690	59.50	0.000	
Days*Diet						
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 10a, 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	84.67 ± 2.52	94.50 ± 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	16.50 ± 6.54
8	79.67 ± 1.26	89.17 ± 1.89	85.33 ± 9.75	11.50 ± 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	9.33 ± 2.93

Table 28b. Growth data (mm TL) for Figures 11a, 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	1.43 ± 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	4.57 ± 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. Percentage nutritional and fatty acid composition of the nematode diets used in first week of the present experiment to feed *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	19.93
Monounsaturates (%)	22.61	20.85	27.83
Polyunsaturates (%)	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. Percentage nutritional and fatty acid composition of the nematode diets used in second week of the present experiment to feed *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
Monounsaturates (%)	32.05	29.37	33.5
Polyunsaturates (%)	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

DISCUSSION

Nematodes with algal co-feeds

It is known that once internal food resources have been depleted, PZ1 larvae require adequate external food. During this critical first feeding stage (Preston, 1985b), providing algae together with nematodes may support better larval growth, survival and development. In the current study, slower larval development and growth on the live nematode-only diets were significantly improved when a small amount of live algae was fed to *P. indicus* larvae for short periods together with the nematodes. When 30 cells μl^{-1} of live algae (for only 24 h) in addition to lipid-enriched nematodes (EN) were fed to PZ1 larvae, larval survival was improved from 42 % to 72 % and larval total length was also increased from 5.16 to 5.68 mm at PL1/2 stages (Table 1). The algal co-feed appeared to influence the growth during protozoeal stages with significantly ($P<0.001$) better growth rate (0.623 mm day^{-1}) than larvae fed only on the live nematodes (0.505 mm day^{-1}). Similarly, when three different algal co-feeds in addition to EN nematodes were fed to PZ1 larvae in Experiment 2, it was found that the algal co-feeds not only gave higher survival rate, but also better larval growth rates (0.831-0.927 mm day^{-1}) than mixed algal control treatment during protozoeal stages (see Figures 4a and 6a). Wilkenfeld et al., (1984) also obtained better survival, growth and larval development when they fed penaeid larvae on nematodes/algae compared to live nematodes alone.

The nematodes with algal co-feeds promoted better larval survivals (38-63 %) but smaller total lengths (5.63-5.95 mm TL) at PL stages compared to algae/*Artemia* control treatment (32 % and 7.49 mm TL). Growth rates of the larvae fed on the nematodes either with or without algae (except EN/Alg2) did not, however, differ significantly during mysis stages suggesting that once administration of algae as a co-feed is stopped, its positive effect on larval growth progressively decreases. Although it is difficult to

eliminate the idea that the algae might have contributed to the larvae nutritionally as discussed in Chapter 5, improvements in larval growth and survival are likely to be due to an enhanced digestion as a result of higher levels of digestive enzymes induced by the algae (Kumlu et al., 1992; Jones et al., 1993; Le Vay et al., 1993). Figure 9 demonstrates that PZ1 stage larvae fed nematodes together with 15 cells μl^{-1} for 48 h had significantly ($P<0.05$) higher trypsin activity ($larva^{-1}$ and μg^{-1} dry weight) than those fed live nematodes only. Highest trypsin activity was induced by the mixed algae ($P<0.05$). Data in Chapter 5 also suggest that algae at only 15 cells μl^{-1} along with microencapsulated artificial diets play a role in stimulating trypsin activity in *P. indicus* larvae thus improving larval survival and growth comparable to a control diet of mixed algae and *Artemia*. As with artificial diets, the nematodes may be relatively indigestible (Hofsten et al., 1983) in comparison to *Artemia*, and may lack substances to trigger digestive enzymes at levels stimulated by algae. Hence based on the present results and those obtained by Wilkenfeld et al., (1984) and Biedenbach et al., (1989) it may, under commercial conditions, be useful to feed penaeid larvae on a combination of algae and nematodes to produce larval growth and survival equal, if not better, to an algae/*Artemia* feeding regime. It should be noted that in addition to the role as a gut enzyme stimulant, an algal co-feed may also correct a possible nutritional deficiency or imbalance of the nematodes (Biedenbach et al., 1989). For example, when the algae (30 cells μl^{-1}) was provided with EN nematodes in Experiment 1, the abnormal 'L' shaped specimens observed in the larvae fed on the EN nematodes alone were not seen.

Effect of lipid-enrichment on larval growth, survival and development

Biochemical composition of the nematode *P. redivivus* (Tables 13, 21 and 29) and *Artemia* (Biedenbach et al., 1989; Le Vay et al., 1993) reveals that both animal feeds contain similar levels of protein and lipids. Little is known about absolute nutritional requirements of penaeid larvae

unexpected mortalities may be attributed to an over high HUFA content or to incorrect balance of the fatty acid contents of the nematodes (Rees et al., 1994). For example, when two nematode feeds (EN and PEN) enriched with the same fish oil (capelin) were fed to *P. indicus* larvae, the EN nematode caused a very high larval mortality rate (12 % day^{-1}) resulting in only 10% survival at metamorphosis, whereas the other enriched-nematodes with the same lipid source (pigmented lipid-enriched = PEN) or EN/ Alg1, 2, 3 (lipid-enriched nematodes with algal co-feeds) gave low mortality rates. The main differences between the nutritional content of the two nematode feeds were their lipid and HUFA contents (EPA and DHA). The PEN nematodes had significantly higher lipid (33 %) and DHA whereas the EN nematodes had double the level of EPA. Millamena et al., (1988) found poor growth in *P. monodon* postlarvae reared on rice bran-fed *Artemia* which contained abnormal amounts of 18: 2ω-6 fatty acid. The abnormal larval development on EN nematodes observed in Experiment 1 was not seen with EN nematodes plus 30 cells μl^{-1} of live algae for only 24 h. It appears that the imbalanced nutritional content of the EN nematodes was compensated by the algal co-feed. Due to inferior growth and survivals obtained from the larvae fed capelin lipid-enriched nematodes, it became necessary to compare other lipid sources that are known to contain high levels of HUFA.

The biochemical composition of the nematodes, which were enriched with three different lipid sources and used to feed *P. indicus* larvae throughout all larval stages (Experiment 3), is given in Tables 21a, b. In agreement with Rouse et al., (1992), the present results suggest that lipid and more specifically the fatty acid content of the nematodes can be manipulated by using different culture media. Lipid-enrichment caused considerable decrease in protein content of the nematodes from 74.4-77.0 (NEN) to 52.9-64.6 % (EN), 55.4-59.1 % (MAR) and to 51.9-56.7 (CLO) and slight increase in the lipid levels (see Tables 21a, b). Within the fatty acid fraction

(Kanazawa, 1985; 1990). This depends on species, age and feeding habit, water temperature, energy content and dietary protein sources of a diet. Optimal protein, carbohydrate and lipid requirement for penaeids may be 23-57 %, 15-25 % and 6-10 % of the diet respectively (Kanazawa, 1990; Chen, 1993). Colvin (1976) reports that 43 % protein was optimal for *P. indicus*. Non-lipid-enriched (NEN) nematodes used in the present study contained 66-77 % protein and 14.1-14.8 % lipid. Amino-acid composition of *Panagrellus* sp., is reported to be similar to that of *Artemia* (Kahan et al., 1980). Protein and lipid levels of *P. redivivus* used to feed *P. vannamei* were 48.3 % and 17.3 % respectively (Biedenbach et al., 1989). Macronutrients of the nematodes used in the present study were well within the range required by penaeid larvae. However, the fatty acid fraction of the lipid of the nematodes showed that particularly the ω-3 series of the HUFA may not be sufficient to promote good growth and survival in *P. indicus*. Recent studies have shown that these HUFA play an important role in penaeid nutrition (Léger and Sorgeloos, 1992; Rees et al., 1994) and may be essential in the diets since marine shrimps have poor ability to synthesise these fatty acids (Jones et al., 1979a; Kanazawa et al., 1979). Xu et al., (1993) compared 18: 2ω-6, 18:3 ω-3, 20: 4ω-6 and 22: 6ω-3 fatty acids and concluded that a diet containing 1% of 22: 6ω-3 promoted the highest survival, moulting frequency and wet-weight gain in *P. chinensis* juveniles. These authors suggest that highest essential fatty acid (EFA) values decrease in the order of 22: 6ω-3>20: 4ω-6>18: 3ω-3>18: 2ω-6. In the present study, ω-3 series of the HUFA especially 20: 5ω-3 (eicosapentaenoic acid: EPA) and 22: 6ω-3 (docosahexaenoic acid: DHA) content of the NEN nematodes were only 1.29-1.97% and 0-0.8% respectively (Tables 25, 33).

Results of Experiments 1 and 2 show that despite lipid-enrichment (with capelin oil) of the nematodes, which increased the HUFA, particularly 20: 5ω-3 and 22: 6ω-3 (see Table 13), heavy larval mortalities and abnormal development were obtained with the lipid-enriched (EN) nematodes. These

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of the lipids, the lipid-enrichment increased the ω-3 series of the 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). Among ω-3 fatty acids, EPA were 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). Ratio of ω-6/ω-3 fatty acids in NEN nematodes was 6-7 times higher than the lipid-enriched nematodes. For the ω-6 series, the DHA of the lipid enriched nematodes was significantly improved from 0-0.8 % (NEN) to 3.91-4.0 % (EN), 3.74-4.39 % (MAR) and to 2.79-2.86 %. From the results of Tables 21a, b it appears that biochemical content of the nematodes during larval culture of *P. indicus* did not differ greatly. The nutritional value of lipids in nutrition of crustaceans has been shown to be based on the type and content of the long chain unsaturated fatty acids (Xu et al., 1993). Work by Millamena et al., (1988) has shown that feeding *Artemia* nauplii with increased HUFA levels results in better growth and survivals in *P. monodon* larvae.

In the current investigation, when the nematodes enriched with different lipid sources were fed to *P. indicus* larvae, the lowest mortality rate was found in the larvae fed the CLO nematodes (2.3 % day^{-1}) during protozoeal stages (Figure 8a). Larvae fed MAR and EN nematodes also displayed significantly lower mortality rates (3.97-5.40 % day^{-1}) compared to those fed NEN nematodes (7.55 % day^{-1}) and control live mixed algae (6.56% day^{-1}). Larvae receiving the lipid-enriched nematodes had significantly higher survivals (69-77 %) than both NEN nematodes (53.5 %) and the control diet (54.83 %) until PL stages. Larvae fed on EN nematodes exhibited equal growth rate (0.574 mm day^{-1}) to those fed live mixed algae (0.576 mm day^{-1}) between PZ1 and PZ3/M1 stages. As a result, it appears that CLO is a suitable lipid source to improve survival whereas capelin oil and marilla oil favours growth of *P. indicus* larvae. Larvae fed lipid-enriched nematodes developed into M1 and PL1 stages one day earlier than those fed non-lipid enriched nematodes. Studies with *P. japonicus* (Ka-

nazawa et al., 1977; 1979), *P. indicus* (Read, 1981) and *P. chinensis* (Xu et al., 1993) showed that 18: 3ω-3 had greater essential fatty acid (EFA) value than 18: 2ω-6. But, these authors also found that 20: 5ω-3 and 22: 6ω-3 had the greatest EFA value for marine crustaceans. Xu et al., (1993) report that arachidonic acid (20: 4ω-6) has an EFA value of between 18: 3ω-3 and 22: 6ω3 and concluded that this HUFA may play an important nutritional role for penaeids. Since there was not considerable difference in other fatty acids, and a decrease existed in the level of arachidonic acid, in lipid-enriched nematodes, the better larval performance obtained with lipid-enriched nematodes may be attributable to the presence of high levels of C20: 5ω-3 and C22: 6ω-3 of the HUFA (see Experiment 3). Levine and Sulkin (1984) also report enhanced larval survival and development on diets rich in DHA and suggest that this HUFA and EPA are important in promoting successful development to megalopa in a brachyuran crab, *Eurypanopeus depressus*. Discrepancies in present results may not only be due to different levels of some important fatty acids, but also to their incorrect balance in the nematodes during larval culture (Rees et al., 1994). When Read (1981) included linoleic and linolenic acids using anchovy oil into a purified diet, he found an improvement in both growth and survival in *P. indicus* juveniles. This author concluded that *P. indicus* has limited capacity to elongate and desaturate linoleic and linolenic acids to C20 and C22 fatty acids and shows a similar qualitative fatty acid requirement to other penaeid species.

Effect of astaxanthin on larval pigmentation, growth and survival

Pigmentation

If nematodes are to be used to totally replace the conventional live diets in commercial penaeid hatcheries, the pale colour of the postlarvae (PL) normally obtained with nematode feeds must be rectified. In commercial hatcheries, where the PL's are produced for on-growing farms, the market value of PL's may be related directly to the intensity of the body colour. PL's

pigmented at similar levels to those fed a standard algae/*Artemia* may show more resistance to strong light and low oxygen levels which may occur during or after the release of the PL's into on-growing ponds. Photoprotective and respiratory functions of the carotenoids during egg and larval development in fish and crustaceans have been suggested in the literature (Tacon, 1981; Torrissen, 1989; Bird and Savage, 1990; Funk and Hobson, 1991; Estermann, 1994). Chien and Jeng (1992) suggest that colour is one of the major factors influencing the price of live Kuruma prawn, *P. japonicus*, in the Japanese market. It is known that astaxanthin is the main pigment associated with colour in prawns and it is the pigment most easily utilised for this purpose (Katayama et al., 1971; D'Abramo et al., 1983). Since crustaceans are incapable of synthesising carotenoids, these pigments have to be supplied as dietary ingredients (Latscha, 1990, Estermann, 1994). The nematode, *P. redivivus*, can be used as a carrier to supply astaxanthin (in the gut) to penaeid larvae. A dietary level of 1.5 % (w/w) Carophyll pink (containing 8 % astaxanthin) was found to be optimum to pigment cultured nematodes without lowering the nematode population or wasting the pigment (Fisher, pers. comm.). When grown on a medium containing capelin fish oil, this level of Carophyll pink gave 1.43 µg astaxanthin g^{-1} dry weight of nematodes.

P. indicus has to pass six nauplii, three zoeal and three mysis stages until they reach final metamorphosis. Several carotenoids in large quantities have been reported to exist in the eggs of crustaceans (Kour and Subramoniam, 1992). Penaeid larvae provide the pigments during the non-feeding nauplius stages from the vitellin reserves. At first feeding, the zoea larvae acquire the carotenoids directly from the pigments of the microalgae used to feed the larvae. Involvement of the carotenoids during larval stages may be important as significant concentrations of these pigments are present in the live food organisms such as microalgae, *Artemia*, rotifers etc., used as feed in commercial aquaculture hatcheries (Tacon, 1981). Petit et al.,

(1991) report that zoea stages of *P. japonicus* larvae store the carotenoids in the same form as they are acquired from the algae without transformation. These authors found canthaxanthin in high quantities in the mysis larvae as this pigment was also the major carotenoid of *Artemia* nauplii. In the present study, astaxanthin was used to pigment *P. indicus* during larval development (PZ1 to PL stages) as this pigment can be stored directly and utilised more efficiently in the body (Yamada et al., 1990; Petit et al., 1991; Négre-Sadargues et al., 1993). In a preliminary trial (Experiment 1), when pigmented nematodes were fed to *P. indicus* larvae at PZ1 stage, the effect of pigments on the tail and appendages of the larvae was clearly visible under a microscope after just 24 h of feeding. The pigmented-nematodes obtained from AGC and used in Experiment 1 were extremely dirty with components of the Carophyll Pink product. This caused immediate larval fouling leading to high mortalities during larval culture. Nevertheless, larvae fed the pigmented-nematodes at metamorphosis were distinctly pigmented compared to larvae fed non-pigmented (EN or NEN) nematodes.

Alternative methods of improving colouration in penaeid larvae may be the use of algae as a co-feed in addition to nematode feeding regimes. Present results showed that when algae (*T. chuii*/*S. costatum*) in low cell concentrations (15-30 cells μl^{-1} day^{-1}) were fed to *P. indicus* larvae for short periods such as 24 or 48 h along with the nematodes, the larvae were better pigmented than larvae fed the nematode feeds alone. In Experiment 1 and 2, when algae ceased to be fed after 24-48 h, the colour of the larvae gradually faded until metamorphosis. Therefore, if well pigmented PL's are to be produced, algae as a co-feed in addition to nematodes should be provided to the larvae during all larval stages. Several authors have shown that addition of *Spirulina*, oleoresin paprika, crayfish waste extract, corn gluten into diets improves pigmentation in crustaceans (Tanaka et al., 1976; D'Abramo et al., 1983). Petit et al., (1991), who are the only authors to study pigments in a penaeid species during larval development, stated that

pigment composition of the larvae is dependent on the algal species they feed upon. Beside pigmentation, algal co-feeds will compensate for any possible nutritional deficiency such as vitamins and fatty acids (Rouse et al., 1992), and any imbalance in the nutritional content of the nematodes which may happen as a result of pigment or lipid enrichment (Experiment 1). They will also stimulate larval digestive enzyme activities.

Survival

When clean nematode cultures were supplied in Experiment 2, the pigmented and lipid-enriched nematodes (PEN) not only gave good larval pigmentation but also significantly ($P<0.05$) better larval survival (68 %) over that obtained from the control diet (32 %), NEN nematodes (51 %) and EN nematodes (10.33 %) at metamorphosis (see Table 7). Results from Experiment 4 (Table 34) show that PEN nematodes promoted the lowest mortality (2.83 % day-1) in comparison to PLC (6.30 % day-1), CLO nematodes (4.22 % day-1) and the control diet (17.68 % day-1) during protozoeal stages. The PEN nematodes supported 88 % survival whereas PLC nematodes gave 78 % survival at metamorphosis. These results are in agreement with Yamada et al., (1990) who demonstrated a significantly better survival (91.3 %) on an astaxanthin supplemented diet (100 mg kg-1) than on a basal diet (57.1 %) for *P. japonicus* juveniles (8.5 g). The same authors did not find any notable change in daily feed intake, percent weight gain or feed efficiency in animals fed on either of the diets. Négre-Sadargues et al., (1993) also found the highest survival rate in *P. japonicus* juveniles (12 g) fed an astaxanthin/canthaxanthin (50:50 mg kg-1) containing diet. These authors reported a positive correlation between survival rates and pigment concentration in the prawn tissue and suggested that pigments may play an important role in improving survival of penaeid prawns. The influence of carotenoids on survival of prawns is still obscure (Négre-Sadargues et al., 1993). The functions of pigments on the metabolism of animals, especially fish and crustaceans, remain hypothetical

(Tacon, 1981; Katsuyama and Matsuno, 1988; Torrissen, 1989; Bird and Savage, 1990; Latscha, 1990; Estermann, 1994).

Pigmentation in penaeids is known to depend on animal-related factors (e.g. species, age/stage, sex/maturation, genetics, culture method, water quality and light intensity), pigment-related factors (e.g. type, form, amount and stability of the carotenoids in the feed), and feed-related factors (manufacturing, feed composition, food intake and conversion ratio, bioavailability and administration period) (Latscha, 1990). Results of the present study suggest that although optimum astaxanthin level and the effects of other factors on pigmentation for penaeid larvae are not known, the level used to deliver the pigment to *P. indicus* larvae appears to be effective in improving the colour and survival of the larvae at metamorphosis. The nematode, *P. redivivus*, was observed to retain the pigment in the gut for over six days at 15 °C (Fisher, pers. comm.). D'Abramo et al., (1983) suggest that live *Artemia* fed lobster juveniles (*Homarus americanus*) had the highest pigment level and that this live diet is much more effective in producing pigmentation than a purified diet containing the same carotenoid. Pigments as well as specific factors such as proteolytic enzymes, PUFAs and steroids are susceptible to heating, freezing, freeze-drying, boiling, and air drying, thus use of nematodes to deliver the pigment should be considered seriously for penaeid larvae.

Growth

Despite several experiments conducted to determine any positive effect of the pigment (astaxanthin) on the growth of *P. indicus* larvae in the current study, the results remain unclear. The data in Experiment 1 showed that the larvae fed PEN nematodes displayed better larval growth rate than EN nematodes between PZ1 and PZ3/M1 stages. The larvae fed PNE nematodes, however, had significantly inferior larval growth rate than NEN nematodes during these stages (Table 1). Pigmented-nematode cultures

obtained from AGC were not always clean and this was possibly one of the reasons for contradictory results in this preliminary trial. In Experiment 2, although PEN nematodes promoted better larval growth rate between PZ1 and PZ3/M1 (0.570 mm day^{-1}) than EN or NEN nematodes (0.535-0.540 mm day^{-1}), total lengths at metamorphosis of the larvae on these three diets were not significantly ($P>0.05$) different from each other (Table 7).

Similarly, results of Experiment 4 also showed that PEN nematodes promoted significantly higher larval growth rate (0.455 mm day^{-1}) than either PLC nematodes or CLO (lipid-enriched) nematodes (0.415-0.440 mm day^{-1}) during protozoeal stages. Yet, no significant difference was found between the growth rate of larvae fed PEN and PLC nematodes during mysis stages. Interestingly, the larvae fed on CLO nematodes, in fact, displayed significantly better larval growth rate than those fed the PEN nematodes during these stages (see Table 22). All these results indicate that the pigment has a less pronounced effect on larval growth in comparison to larval survival of *P. indicus* under the experimental conditions used during the present studies. Yamada et al., (1990) also found no significant effect of various pigments (astaxanthin, β-carotene and canthaxanthin) on weight gain in *P. japonicus* juveniles. Similar conclusions were made by Chien and Jeng (1992) who studied the effect of different pigment sources and their various levels on the pigmentation, survival and growth of *P. japonicus*. In another investigation, Négre-Sadargues et al., (1993) could not find any experimental evidence to support any possible influence of astaxanthin, canthaxanthin or the combination of both pigments on the growth of *P. japonicus* juveniles. Hence, the present results suggest that the pigment delivered via nematodes to *P. indicus* may not have any benefit on growth during larval development of this penaeid species

Larval development

Larvae fed algae during herbivorous stages and *Artemia* during mysis and early PL stages always developed faster than those fed nematodes. For ex-

ample, in Experiment 1, when *P. indicus* PZ1 larvae were fed the mixed algae, all the larvae moulted into M1 stage on day 4 while 87.5 % of the larvae fed NEN nematodes were still at PZ3 stage. Again, when these M1 larvae fed on *Artemia* nauplii they metamorphosed into PL4 (15.5 %), while almost half of the larvae on NEN nematodes were still at M3 stages (see Table 5). When the nematodes were fed with algal co-feeds for a short duration, a faster larval development was observed both until M1 and PL stages. Table 11 shows that while NEN and EN nematodes were still at PZ3 stage on the 4th day of the culture, 50-100 % of the larvae fed EN/Alg1, 2, 3 feeding regimes were at M1 stage. It appears that addition of a low amount of algae into the culture together with the nematodes was sufficient to accelerate larval development, possibly as a result of better digestion via stimulated larval digestive enzyme activities by the algae rather than its nutritional contribution (Kumlu et al., 1992; Jones et al., 1993; Le Vay et al., 1993).

The present study suggests that pigmentation of the nematodes does not appear to accelerate larval development of *P. indicus* compared to non-pigmented or placebo pigmented nematodes. Slightly faster larval development observed in Experiment 1 (see Table 5) and Experiment 2 (Table 11) may have resulted from different nutritional content of the nematodes. As a result, addition of low amount of algae throughout of larval stages of *P. indicus* is suggested to obtain larval growth and development comparable to those obtained from live algae/*Artemia* feed regimes. Delivery of astaxanthin via nematodes to pigment *P. indicus* larvae appears to be a successful method. This pigment significantly improves larval survival of this penaeid species compared to non-pigmented nematodes. However, there is no strong evidence to show benefit of pigment additives on larval growth of *P. indicus*.

CHAPTER 5

THE EFFECT OF LIVE AND ARTIFICIAL DIETS ON SURVIVAL, GROWTH, AND TRYPSIN ACTIVITY IN LARVAE OF *Penaeus indicus.*

Parts of this chapter have contributed to the following publications:

1. Kumlu, M., Le Vay, L. and Jones, D. A. 1994. Recent advances in the development of microencapsulated diets for shrimp larval culture. In: Kas, H. S. and Hincal, A. A. (Eds.), Minutes of the 9th Int. Symposium On Microencapsulation. Editions de Santé, Paris. pp. 205-208. (1994).

2. Kumlu, M. and Jones, D. A. 1995. Role of microalgae as a gut enzyme stimulant in rearing *Penaeus indicus* larvae on microencapsulated diets. Book of Abstracts of the World Aquaculture '95' San Diego, February 1-4, 1995. World Aquaculture Society, Baton Rouge, Louisiana, USA. p. 158.

INTRODUCTION

Penaeus indicus is an important commercial penaeid shrimp species cultured mainly on extensive farms in the Southeast Asia and is estimated to account for 5.4-6 % of the total world shrimp production (Rosenberry, 1989; Weidner and Rosenberry, 1992; Csawas, 1994). Despite a slower growth rate in culture conditions compared to other penaeid species such as *P. monodon*, *P. vannamei* (Aquacop, 1984), it is a species of great potential to the shrimp industry as it is tolerant of recirculation systems and readily matures in captivity (Aquacop, 1983; 1984). It is possible to close the life cycle in captivity and to produce disease free seed throughout the year.

Hatchery techniques for *P. indicus* are not yet well established, and only few biological aspects such as ingestion, growth, development, stocking density and energetics have been studied for the larvae of this species on live diets (Emmerson, 1980; 1984; Emmerson and Andrews, 1981). Although there have been numerous attempts to partially and/or completely replace live conventional diets (live microalgae and *Artemia*) with various artificial diets (microbound and microencapsulated diets) for the larval culture of *P. monodon* (Kurmaly et al., 1989a), *P. japonicus* (Kanazawa et al., 1981), *P. vannamei*, and *P. stylirostris* (Jones et al., 1987; Ottogali, 1991), there has only been one previous attempt to rear *P. indicus* on an artificial microbound diet (Galgani and Aquacop, 1988). In their study, the optimal feeding level of this microbound diet was established and live unicellular algae were replaced during the larval zoeal stages only.

Numerous authors have shown that artificial diets fed alone generally promote inferior survival and slower growth in comparison to larvae fed on live diets (Galgani and Aquacop, 1988; Kurmaly et al., 1989a; Kamarudin, 1992). To further improve these diets, recent investigations have focused

on larval digestive morphology, physiology and digestive enzymes of commercially important penaeid shrimp species. Although it is known that the hepatopancreas (HP) is the main production site of digestive enzymes, studies of Lovett and Felder, (1989; 1990a, b) on *P. setiferus* larvae and Abubakr and Jones (1992) with *P. monodon*, have revealed 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 artificial diets is attributed to the relatively high digestive enzyme activity present during early larval stages (Jones and Kurmaly, 1987). However, growth on artificial diets is generally less than optimum and it is thought to be necessary to use highly digestible dietary ingredients and/or diets that stimulate digestive enzyme secretion (Le Vay et al., 1993). Ten cells μl^{-1} of live or even frozen algae significantly enhances trypsin activity during herbivorous larval stages (Amjad et al., 1992; Kumlu et al., 1992) and accelerates the growth of *P. monodon* larvae to that comparable to larvae fed live diets (Kamarudin, 1992). Algae appear to contain substances that trigger larval digestive enzyme activity, or enhance digestion and thus improve survival and growth on artificial diets (Amjad et al., 1992; Jones et al., 1993; Rodríguez et al., 1994).

The present study contributes towards a further understanding of the digestive physiology of *P. indicus* larvae. Complete replacement of live microalgae at protozoeal, and live *Artemia* nauplii at mysis stages with MED are attempted in larval culture of this penaeid species. For the first time, the optimum feeding level of MED, trypsin activity during larval development, influence of MED and a low level of algal co-feed on the digestive enzyme activity, as well as growth and survival of the larvae are investigated. As a gut enzyme stimulant, a microalgae species (*Rhinomonas reticulata*) was incorporated into microcapsules and larval trypsin activities in response to this diet are measured to determine whether the algal substances which stimulate digestive enzymes, are successfully preserved by

the process of microencapsulation. The effect of feeding a low number of *Artemia* in combination with MED was investigated for effects on growth, survival and trypsin activity of mysis larvae to determine whether the prey influences larval enzyme levels by providing a more digestible diet.

MATERIAL AND METHODS

Rearing Procedure

All the *Penaeus indicus* larvae used in present experiments were obtained from broodstock originating from India held in two circular 4 tonne black tanks, at the School of Ocean Sciences, Menai Bridge, UK. After spawning, concentrated eggs were rinsed and stocked in a 50-l tank filled with filtered (0.2 μm) and UV-irradiated sea water. At stage PZ1, the larvae were rinsed again with filtered and UV-irradiated sea water and treated with an antibiotic (0.2 ppm furazolidone). The larvae were stocked at a density of 100 l^{-1} in 2-l round bottom glass flasks to assess survival and growth performance on different live and artificial diets (from two replicates).

The water temperature was maintained at 28 °C by keeping the flasks in a thermostatistically controlled water bath. Culture sea water used in all experiments was adjusted to 25 ppt salinity by adding distilled water (see Chapter 1), and a gentle aeration was maintained through a silicone rubber tube with a glass rod at the tip. Every other day the flasks were emptied and the larvae counted and staged according to Silas et al., (1978). Total length (TL) of 10-13 larvae from each replicate were measured from the tip of their rostrum to the end of telson under a binocular microscope. All artificial diets were hydrated daily and introduced to the larval culture at 08.00, 12.00, 20.00, 24.00 h. Algal species at the School of Ocean Sciences are grown in semi-continuous culture (Walne, 1966). The algal cell density was estimated using a haemocytometer and a Coulter Counter

(Model ZB: Coulter Electronics). Newly hatched (24 h at 28 °C) *Artemia nauplii* (INVE AQUACULTURE, Belgium) were introduced as prey to the larvae fed on the live algae and some artificial diet treatments.

Experiment 1

Different levels (from 4 to 10 mg l^{-1} day^{-1}) of an experimental FRIPPAK MED was tested to determine the optimum feeding level for *P. indicus* larvae particularly during protozoeal stages. The control treatment consisted of 35 cells μl^{-1} *Skeletonema costatum* and 25 cells μl^{-1} *Tetraselmis chuii* and five *Artemia* nauplii ml^{-1} after stage PZ3 (Chapter 1). From results of preliminary trials and microscopic observation, it was decided to use an antibiotic to prevent bacterial infections. Hence, after the eggs were rinsed with filtered and U/V-irradiated sea water, they were treated with an antibiotic (furazolidone 0.2 ppm). Furthermore, a preventative concentration of this antibiotic was used through all zoeal (0.2 ppm) and mysis stages (0.4 ppm) in the culture (Galgani and Aquacop, 1988). To evaluate the effect of the antibiotic, some larvae were fed on the MED without the antibiotic treatment. Survival and growth of the larvae were assessed from two replicates.

Experiment 2

Total trypsin activity and dry weight of *P. indicus,* reared on the live control diet in three 5-l round bottom glass flasks, were determined throughout larval stages (from PZ1 to PL1 stage) as described in General Material and Methods. Specific trypsin activity of the larvae was obtained by dividing total trypsin activity by the dry weight (Table 6) of the larvae.

Experiment 3

The following feeding regimes were used to rear the larvae at this experiment;

Diet A (Control): 35 cells μl^{-1} *S. costatum* and 25 cells μl^{-1} *T. chuii* throughout the larval stages with the addition of five newly hatched *Artemia* ml^{-1} during mysis stages,

Diet B: FRIPPAK experimental MED throughout all larval stages; 8 mg l^{-1} CAR PZ1 to PZ3/M1, 12 mg l^{-1} CD2 from M1 to M3, 16 mg l^{-1} CD3 from M3 to PL1/2,

Diet C: The experimental FRIPPAK diets as above plus 15 cells μl^{-1} (2:1, *S. costatum* + *T. chuii*) frozen algal co-feed from PZ1 to PL1 stages,

Diet D: The experimental FRIPPAK diets plus frozen algal co-feed as Diet C and five *Artemia* ml^{-1} between M1 and PL1,

Diet E: The experimental FRIPPAK diets as Diet B and *Artemia* (five ml^{-1}) from M1 onwards.

Samples for trypsin activity and larval dry weight (only during zoeal stages), were reared in four 5-l, one 10-l and sufficient quantities of 2-l flasks for each diet. The larvae fed MED, MED plus 15 cells frozen mixed algae μl^{-1} and the control live diet were sampled throughout all larval stages. All the larvae reared on Diet A, D and E received five *Artemia* nauplii ml^{-1} from M1 stage onwards. Larvae fed Diet B and C did not receive *Artemia* between M1 and PL stages. Trypsin samples were taken in two replicates for each stage.

Experiment 4

Effect of *Artemia* nauplii ml^{-1} as co-feed with MED was investigated on growth, survival and trypsin activity during mysis stages. For this purpose, the larvae, previously reared on the mixed live algae (control), were fed on:

Diet A: 12 mg l^{-1} MED between stage Z3/M1 and M2 and 16 mg l^{-1} M3 to PL1,

Diet B: the same as Diet A plus one *Artemia* ml^{-1},

Diet C: the same as Diet A plus five *Artemia* ml^{-1}.

Trypsin activity (IU larva^{-1}), survival and total length were assessed from two replicates.

Experiment 5

In this trial, trypsin activity of *P. indicus* larvae fed a control live algae (50 cells µl^{-1} *R. reticulata*), MED and algae incorporated in MED (23 % *R. reticulata*, v/v) was investigated. The diet incorporating algae was produced by inclusion of 23 % of freeze-dried *R. reticulata* into FRIPPAK diet slurry before encapsulation. The artificial diets were fed to the larvae at a level of 8 mg l^{-1} day^{-1} from PZ1 to PZ2 stage. Two replicates for each treatment and three replicates from each sample were used to assess larval trypsin activity.

Statistical Calculations

In the first experiment, one-way ANOVA (Minitab) and appropriate pairwise comparison tests (Tukey's test for equal, and Scheffé's test for unequal observations) were used to compare survival and total length of the larvae at PZ3 and PL1 stages. Survival and growth results in the second experiment were analysed by GLM (General Linear Model) and slopes were calculated for PZ1-PZ3/M1 and PZ3/M1-PL1 to compare the diets with *Artemia* and diets without *Artemia* at mysis stages. All the enzyme results, survival and growth data were compared using one-way or two-way ANOVA and an appropriate multiple pairwise comparison test in Experiments 3, 4 and 5. Prior to any statistical analysis, the data was checked for homogeneity of variances using Bartlett's test. All the statistical calculations were performed in Minitab statistical package.

RESULTS

Experiment 1

In an attempt to totally replace live algae and to determine the best feeding level in the culture of *P. indicus,* four different levels (4-10 mg l^{-1}) of an experimental MED were tested and the results are summarised in Table 1. At 28 °C and 25 ppt salinity, *P. indicus* larvae fed the control diet started to metamorphose into PL1 stage on the 8th day of the culture, whereas MED only fed larvae reached this stage on the 11-12th day of the culture. Statistical results on larval growth and survival at PZ3 and PL1 stages are shown in Tables 2-5. The control diet clearly gave significantly higher survival, maximum growth and faster development from PZ1 to PZ3 (78.50 %, 3.44 mm TL) with a final survival of 68.5 % (at PL1) in 8-9 days ($P<0.05$). Among the four levels of MED tested, 6-8 mg l^{-1} gave significantly better survival ($P<0.05$) than other levels (Table 1). 10 mg l^{-1} MED fouled the larvae and resulted in the lowest survival at the end of the experiment (40 %). Survivals of larvae fed exclusively 8 mg l^{-1} MED during protozoeal stages (PZ1-PZ3/M1) and in conjunction with five *Artemia* nauplii ml^{-1} during subsequent stages (PZ3/M1-PL1) were 67 % and 58 % respectively. The larvae reared on 8 mg l^{-1} MED without the antibiotic died on the 5th day of the culture indicating that application of an antibiotic was necessary.

Experiment 2

Results from this experiment (Figure 1 and Table 7) demonstrate significant differences in trypsin activity during larval development of *P. indicus* fed the live diets. The larvae exhibited a low level of total trypsin ($larva^{-1}$) and specific trypsin (μg^{-1} larval dry weight) activity during early protozoeal stages, but the levels sharply increased and reached a peak at M1 (129.00×10^{-4} IU $organism^{-1}$ and 73.904×10^{-5} IU μg^{-1} dry weight). From this stage onwards, larval trypsin levels decreased until PL1 stage (see Figure 1).

Specific trypsin activity results suggest that *P. indicus* larvae show a low level of this digestive enzyme at PZ1 (9.25 x 10^{-5} IU μg^{-1} dry weight) and PL1 (6.66 x 10^{-5} IU μg^{-1} dry weight) stages. Body dry weight of *P. indicus* during larval development is given in Table 6.

Experiment 3

This experiment investigates the possibility of enhancing the digestive capability of *P. indicus* larvae using a low level of frozen algae as a gut enzyme stimulant and hence improving growth and survival. Complete replacement of both algae and *Artemia* nauplii were also attempted. In the results, the slopes of the treatments were separately compared from PZ1 to PZ3/M1 (protozoeal stages) and from PZ3/M1 to PL1 (mysis stages) in order to compare changes in growth rate, survival rate and trypsin activities as a response to diet composition.

Statistical results on larval growth and survival are presented in Tables 8-11. Figure 2a clearly demonstrates that when only 15 cells μl^{-1} mixed frozen algae was used as a supplemental co-feed with MED, survivals of the larvae were improved to levels (85-91 % final survival at PZ3/M1) equal to that of live algae (91.5%) during protozoeal stages. Larval mortality rates on MED+algal co-feeds (1.63-1.81 % day^{-1}) and the control (1.06 % day^{-1}) were significantly lower than that on MED (7.63-8.75 % day^{-1}) during protozoeal stages (see Tables 8a, b). Survivals of MED only fed larvae (between 46 and 55 %) were significantly inferior to those obtained from MED plus algae fed larvae during this period. However, growth rates of frozen algal additive fed larvae (0.55-0.56 mm day^{-1} TL) were still slower than the mixed algal control (0.79 mm day^{-1}) but were again significantly ($P<0.001$) higher than those (0.42-0.46 mm day^{-1}) obtained from MED only fed larvae (Tables 10a, b).

From PZ3/M1 onwards, some larval treatments received *Artemia* nauplii in addition to the diets (8 mg l^{-1}) whereas others were exclusively fed only MED and MED plus 15 cells μl^{-1} algae until PL1 stage. Figures 2a, b show that addition of *Artemia* to the culture significantly ($P<0.01$) promoted a higher survival rate and growth rate (see Table 11). MED with frozen algal co-feed plus *Artemia* after PZ3 stage gave equal survival (81 %), but slower larval development and an inferior mean total length (7.4 mm) compared to live fed controls (9.65 mm) at the end of the experiment. Final survival of MED only fed larvae and MED plus algae without *Artemia* at PL1 were 36.5% and 65.5 % respectively. These results demonstrate that *P. indicus* larvae can be reared on the MED, as an algal replacement, with a 49 % survival, but that the addition of 15 cells μl^{-1} of frozen algae promotes a 91 % survival.

Figure 3 and two-way ANOVA (Tables 12a, b) demonstrate that the live algae always gave the highest total trypsin activity ($P<0.05$) during protozoeal stages whereas MED as sole diet gave the lowest activity. Live algae induced the highest trypsin activity per larva during herbivoral stages ($P<0.05$). Since there were significant interactions between diets and stages ($P<0.05$), the effects of diets on total and specific trypsin activity were further analysed using one-way ANOVA and Tukey's test for each individual protozoeal larval stage. Any significant effect is summarised as superscripts in Tables 13 and 15. During protozoeal stages (PZ1-PZ3), the larvae fed MED+algae (Diet C) displayed intermediate total trypsin activity between the larvae fed live algae (Diet A) and MED (Diet B) as a sole diet (Table 13). At M1 stage, although MED+algae fed larvae produced higher trypsin activity (84.97×10^{-4} IU $larva^{-1}$) than MED fed ones (75.87×10^{-4} IU $larva^{-1}$), the levels were not significantly different from each other ($P> 0.05$). Tables 14a, b show that specific trypsin activity of larvae fed either live algae or MED plus algal co-feed did not significantly differ from each other between PZ1 and M1 stages ($P>0.05$). Larvae fed MED as a sole diet

displayed the lowest trypsin activity μg^{-1} DW during these stages. Further one-way analyses of variance performed for each individual larval stage indicate that specific trypsin activities of *P. indicus* larvae fed live algae and MED plus frozen algae at PZ1 or PZ2 were not significantly different (see Table 15). At PZ3 stage, however, the larvae fed MED+algae showed higher trypsin activity than those fed algae, whereas this was reversed at M1 stage ($P<0.05$). The frozen algal co-feed (15 cells μl^{-1}) with MED induced significantly more specific trypsin activity at PZ1, PZ2 and PZ3 stages in comparison to those fed MED as a single diet ($P<0.05$). At M1 stage, however, trypsin activities of the larvae fed either diets were not significantly different from each other ($P>0.05$). Dry weight of the larvae between PZ1 and M1 stages is given in Table 16.

Figure 3 shows that the larvae fed solely on MED (Diet B) and/or MED plus frozen algae without *Artemia* (Diet C) maintained higher tryptic activities in comparison to those that received the diets plus five *Artemia* ml^{-1} during mysis stages (Tables 17a, b). The algal additive did not induce significantly higher trypsin activity than MED alone during these stages ($P>0.05$). Table 18 shows any significance in total trypsin activity of *P. indicus* larvae at each larval stage between M2 and PL1. Larvae fed on live algae plus five *Artemia* ml^{-1} showed significantly higher trypsin activity ($P<0.05$) at PL1 stage than those fed the diets plus *Artemia* (Diet D and Diet E).

Experiment 4

The effect of MED (Diet A), MED plus one (Diet B) and MED plus five *Artemia* (Diet C) nauplii ml^{-1} on larval trypsin activity, growth and survival were investigated in this experiment. Although the mysis larvae fed on the MED displayed the highest trypsin activity between M1 and PL1 stages (Figure 5), Tables 19-20 show that this diet resulted in the lowest survival (30 %) and shortest total length (5.34 mm) at PL1 stage (see Figure 4a).

When only one *Artemia* ml^{-1} was offered to the larvae in addition to the MED, larval trypsin activity dropped significantly ($P<0.05$) compared to that of MED only fed larvae (Figure 5) and intermediate growth (5.74 mm) and survival (54 %) resulted (Figures 4a, b and Tables 19-20). A higher concentration of *Artemia* nauplii (5 ml^{-1}) fed together with the MED induced the lowest level of enzyme (Figure 5), but gave the highest survival (73.5 %) and significantly greater ($P<0.05$) total length (5.94 mm) at PL1 stage (Figures 4a, b).

Experiment 5

Figure 6 shows that trypsin activity levels of the larvae fed from PZ1 to PZ2 on live algae (Diet A) and encapsulated diets containing algae (Diet C) were not significantly different ($P>0.05$). The MED (Diet B) alone gave a significantly ($P<0.05$) lower level of trypsin activity than algae included MED.

Table 1. Percentage survival, mean total length (mm) and duration of larval period (days) at PZ3 and PL1 stage of *P. indicus* larvae fed various levels of microencapsulated diets (MED) from PZ1 to PL1. Each value of total length and survival represents a mean ± s.d., (n=2). Means marked with the same superscripts are not significantly different (P>0.05). Each treatment received five *Artemia* nauplii ml^{-1} from PZ3/M1 onwards.

Treatments	Survival %		Total length (mm)		Duration (days)	
	at PZ3	at PL1	at PZ3	at PL1	to PZ3/M1	to PL1
Control	$78.50^{a} \pm 1.41$	$68.50^{a} \pm 4.24$	$3.44^{a} \pm 0.11$	$6.85^{a} \pm 0.32$	4	8-9
MED-4 mg	$59.25^{d} \pm 4.60$	$43.25^{d} \pm 0.35$	$2.99^{b} \pm 0.13$	$5.21^{d} \pm 0.26$	6	12-13
MED-6 mg	$71.25^{b} \pm 1.77$	$53.00^{c} \pm 4.95$	$3.03^{b} \pm 0.14$	$5.33^{d} \pm 0.29$	5-6	12-13
MED-8 mg	$66.75^{c} \pm 0.35$	$58.25^{b} \pm 1.06$	$3.09^{b} \pm 0.11$	$5.52^{c} \pm 0.27$	5-6	12
MED-10 mg	$56.00^{d} \pm 2.83$	$40.00^{d} \pm 2.83$	$3.05^{b} \pm 0.16$	$5.72^{b} \pm 0.28$	5-6	11-12

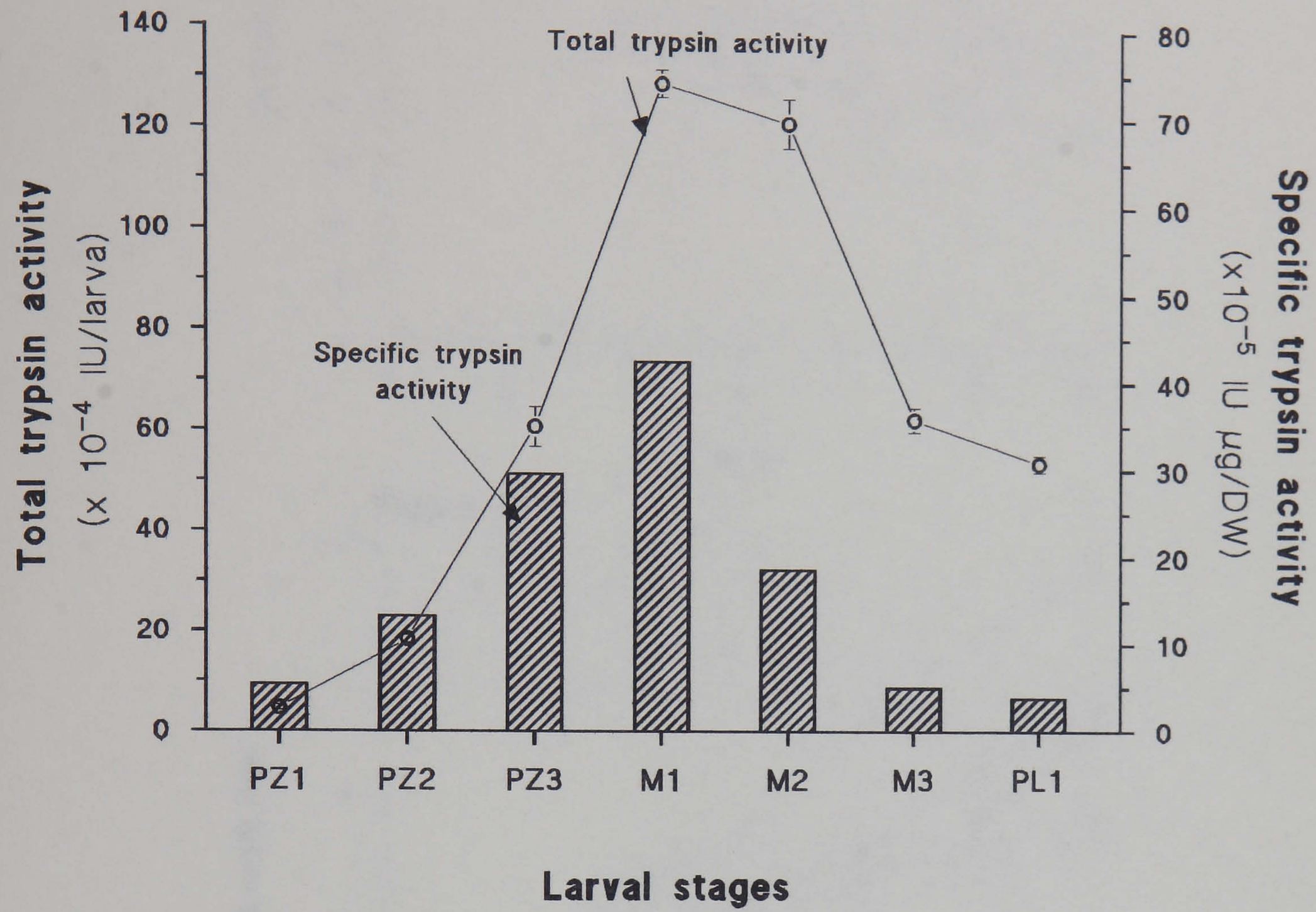


Figure 1. Total and specific trypsin activity in *P. indicus* larvae reared on live control diet (*S. costatum* / *T. chuii* plus *Artemia* PZ3/M1 onwards) from PZ1 to PL1 stage. Each value is a mean ± s.d., n=2.

Figure 2a

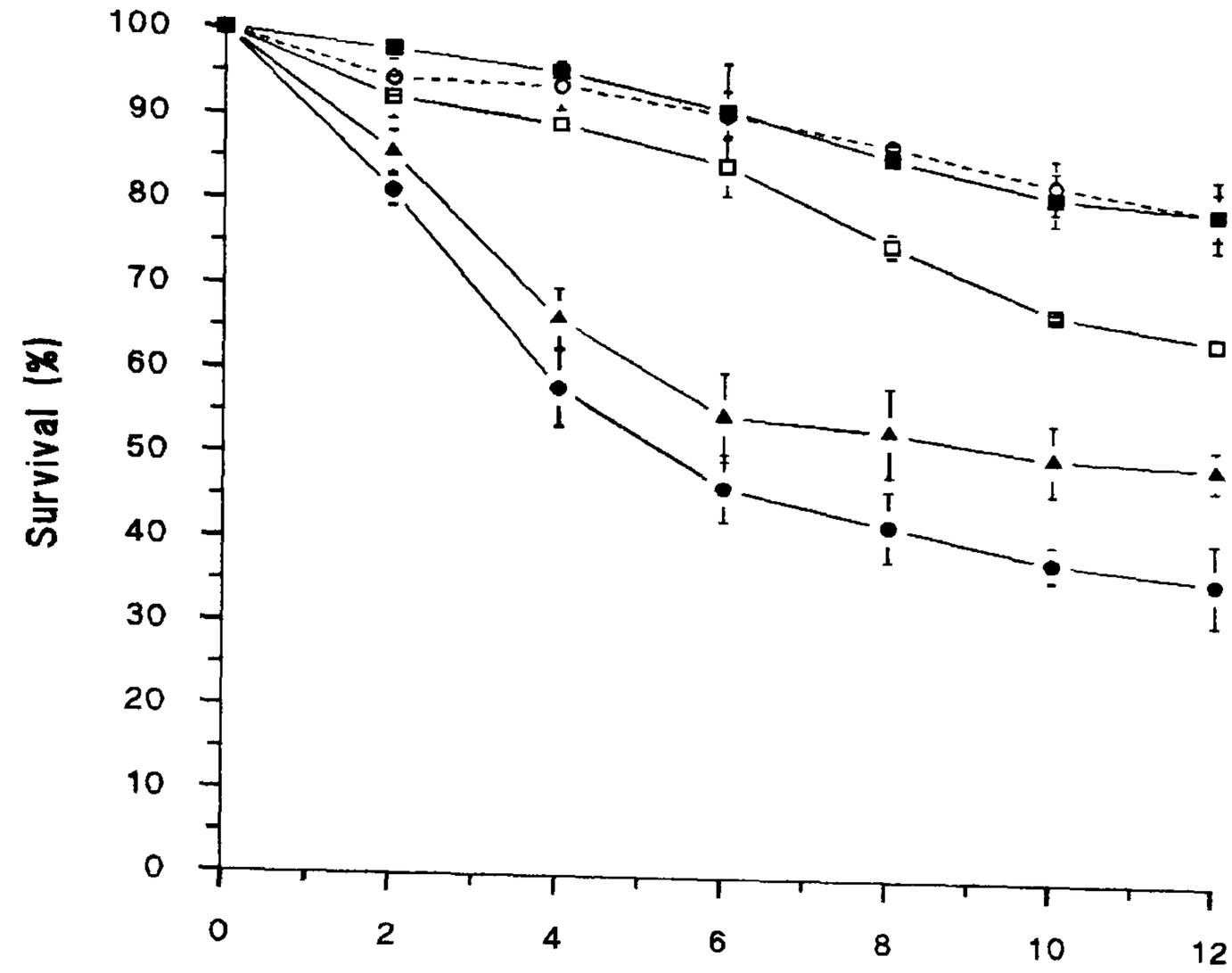


Figure 2b

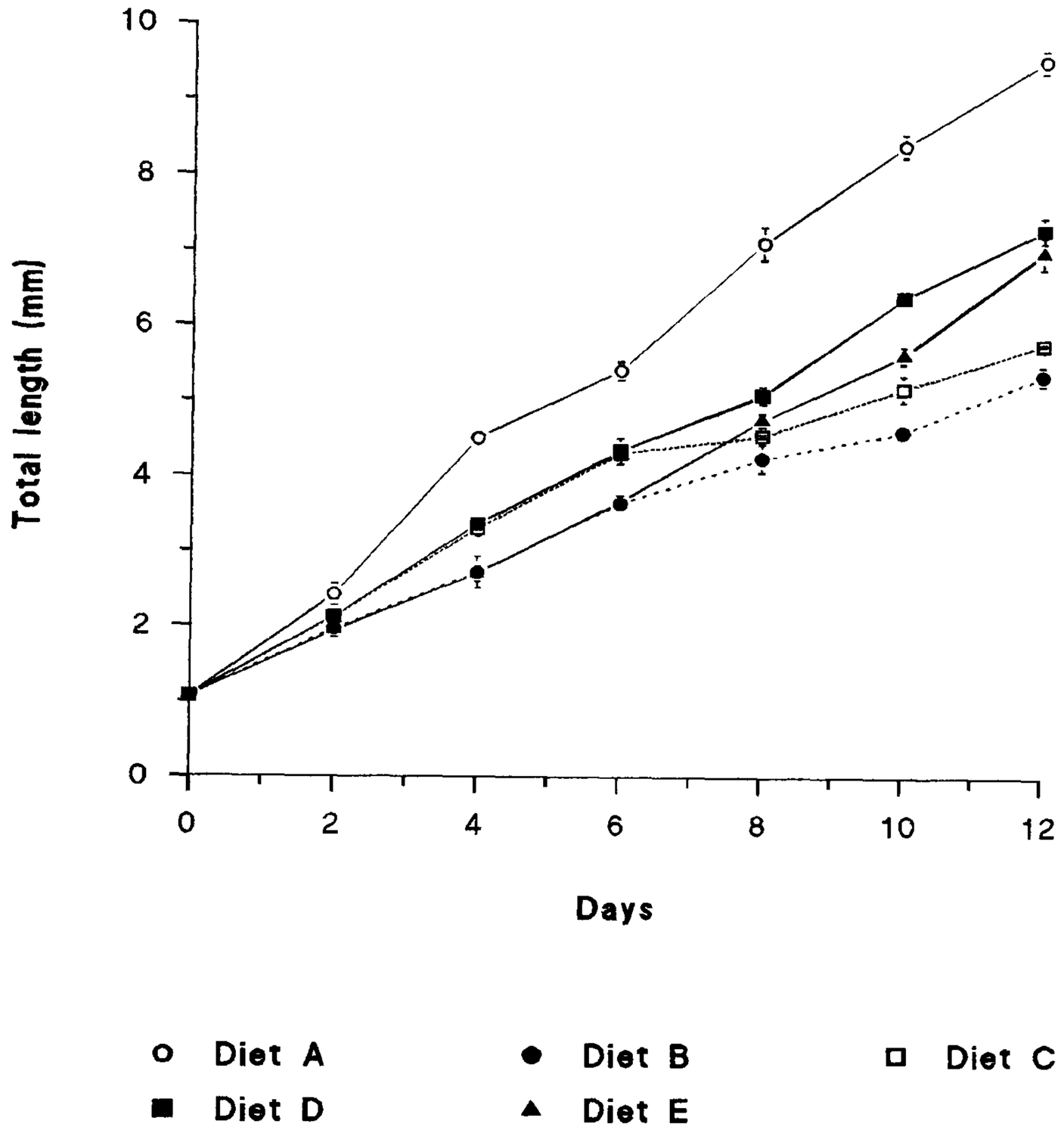


Figure 2. (a) Survival and (b) total length of larvae fed on different feeding regimes from PZ1 to PL1. Each value refers to a mean ± s.d., n=2. Diet A (live mixed alga throughout all larval stages plus five *Artemia* ml^{-1} after PZ3/M1), Diet B (microencapsulated diet= MED throughout all larval stages), Diet C (MED plus 15 cells μl^{-1} frozen algae throughout all stages), Diet D (as diet C plus five *Artemia* ml^{-1} PZ3/M1 onwards), Diet E (as Diet B plus five *Artemia* ml^{-1} during mysis and postlarval stages).

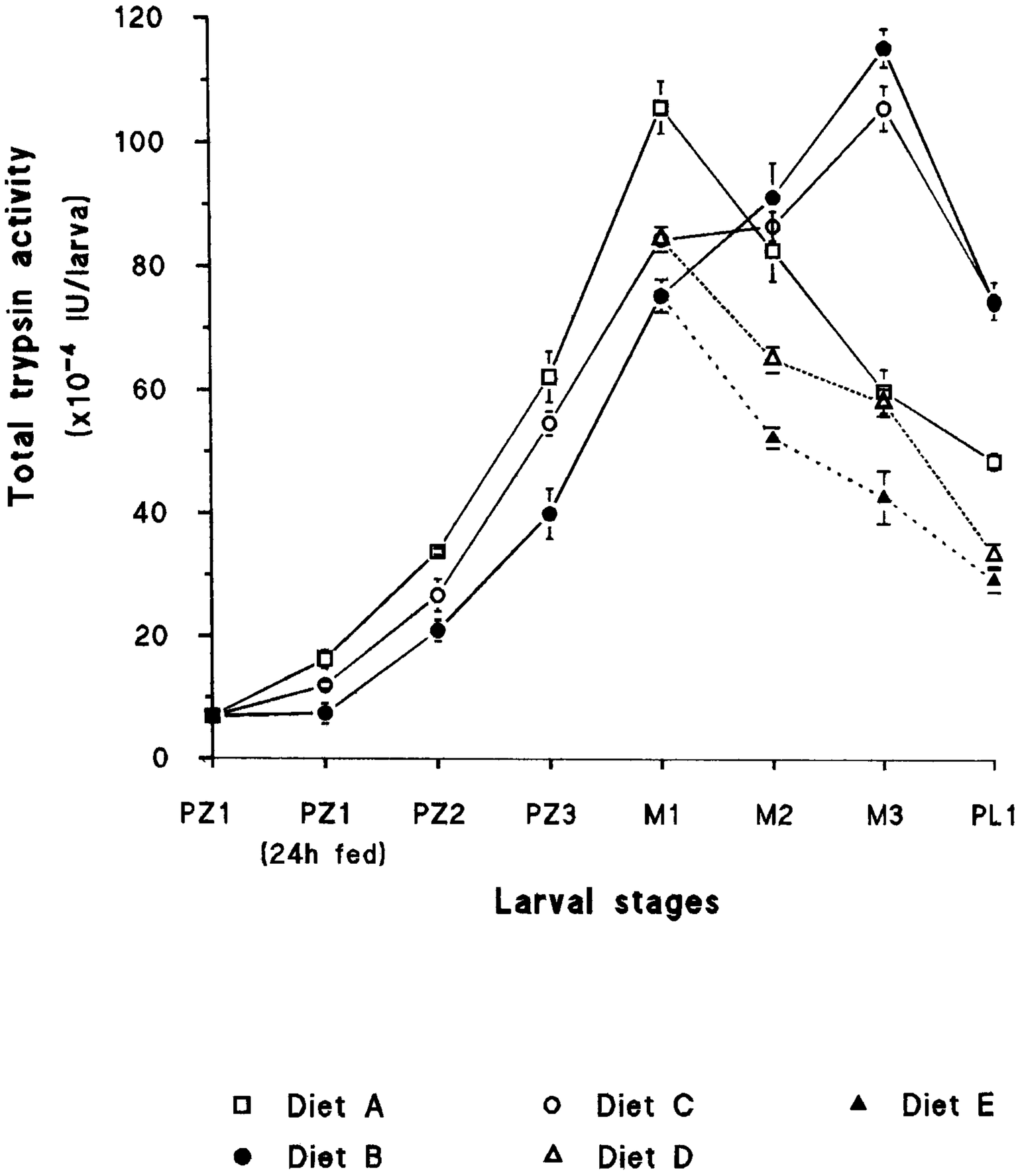


Figure 3. Total trypsin activity of *P. indicus* larvae reared on different feeding regimes throughout all larval stages. Values are means ± s.d., n= 2 for each treatment and n=3 for each assay. Diet A (live mixed algae throughout larval stages plus five *Artemia* ml^{-1} between M1 and PL1), Diet B (microencapsulated diet = MED as a sole diet throughout stages), Diet C (MED plus 15 cells μl^{-1} frozen algae through all larval stages), Diet D (as diet C plus five *Artemia* ml^{-1} between M1 and PL1), Diet E (as Diet B plus *Artemia* between mysis and postlarval stages).

Figure 4a

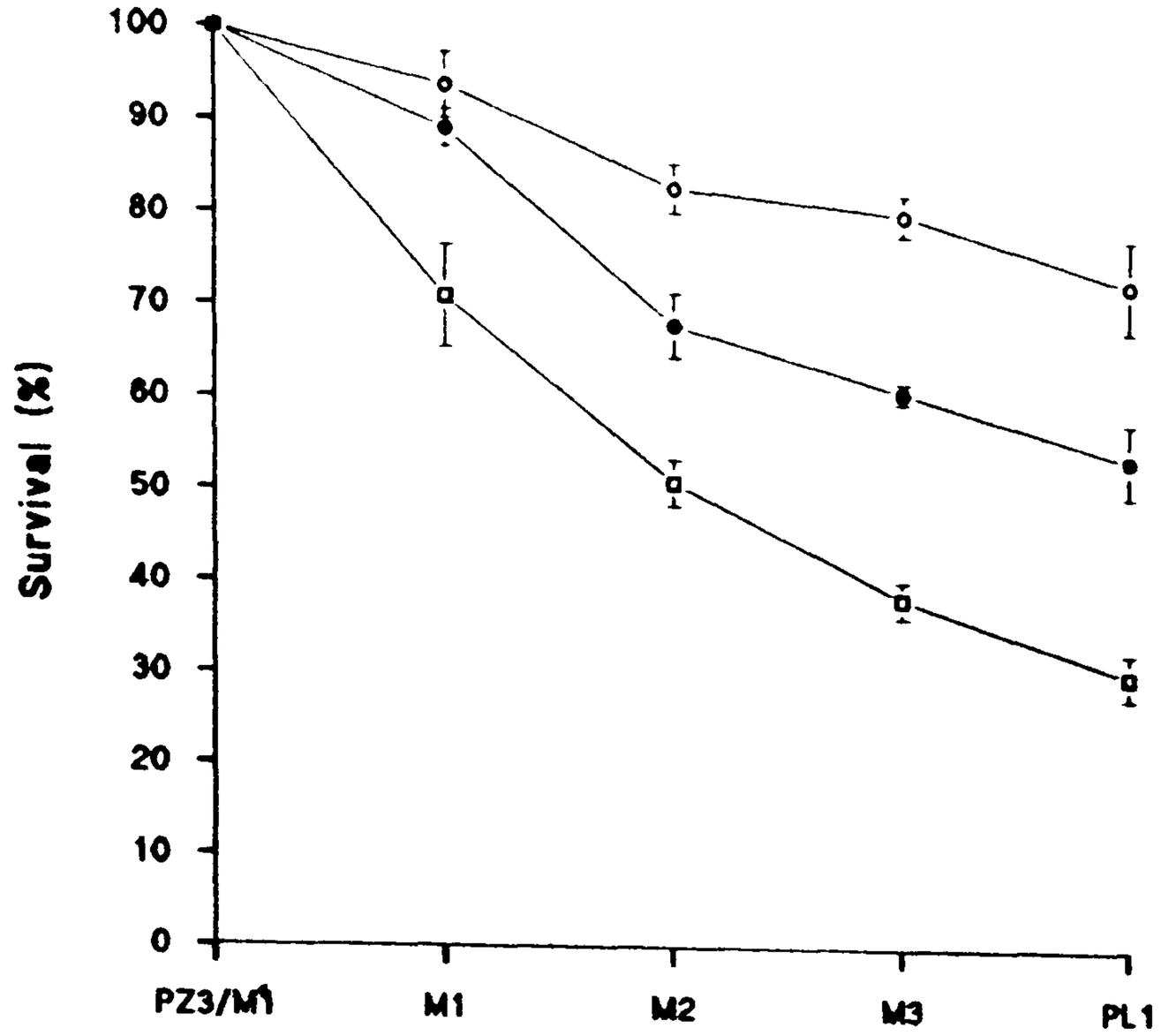
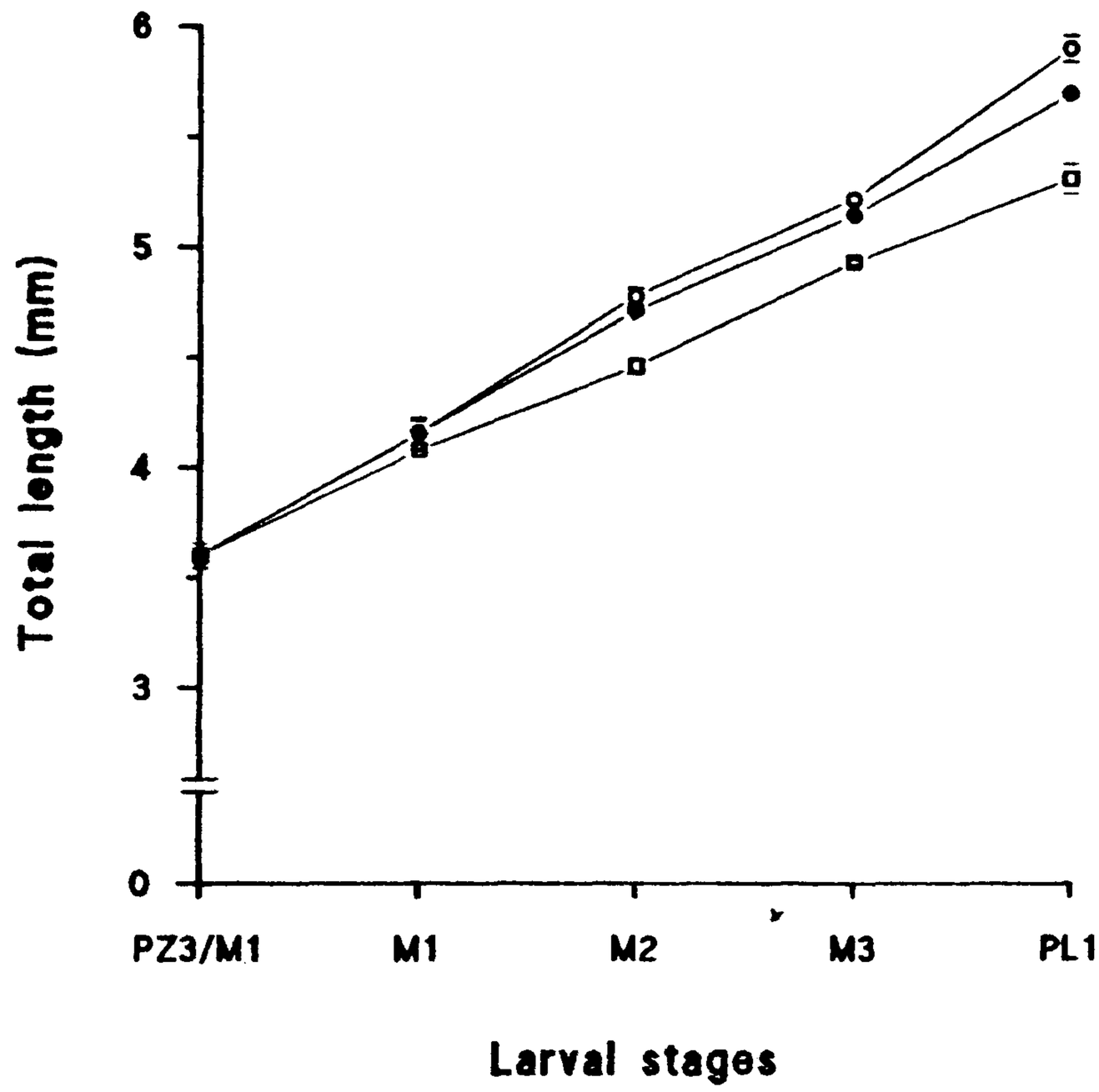


Figure 4b



□ Diet A ● Diet B o Diet C

Figure 4. (a) Survival (%) and (b) growth (mm) of *P. indicus* larvae fed on Diet A (MED), Diet B (MED plus one *Artemia* ml^{-1}), Diet C (MED plus five *Artemia* ml^{-1}) from PZ3/M1 to PL1 stages. Values are means ± s.d., n=2.

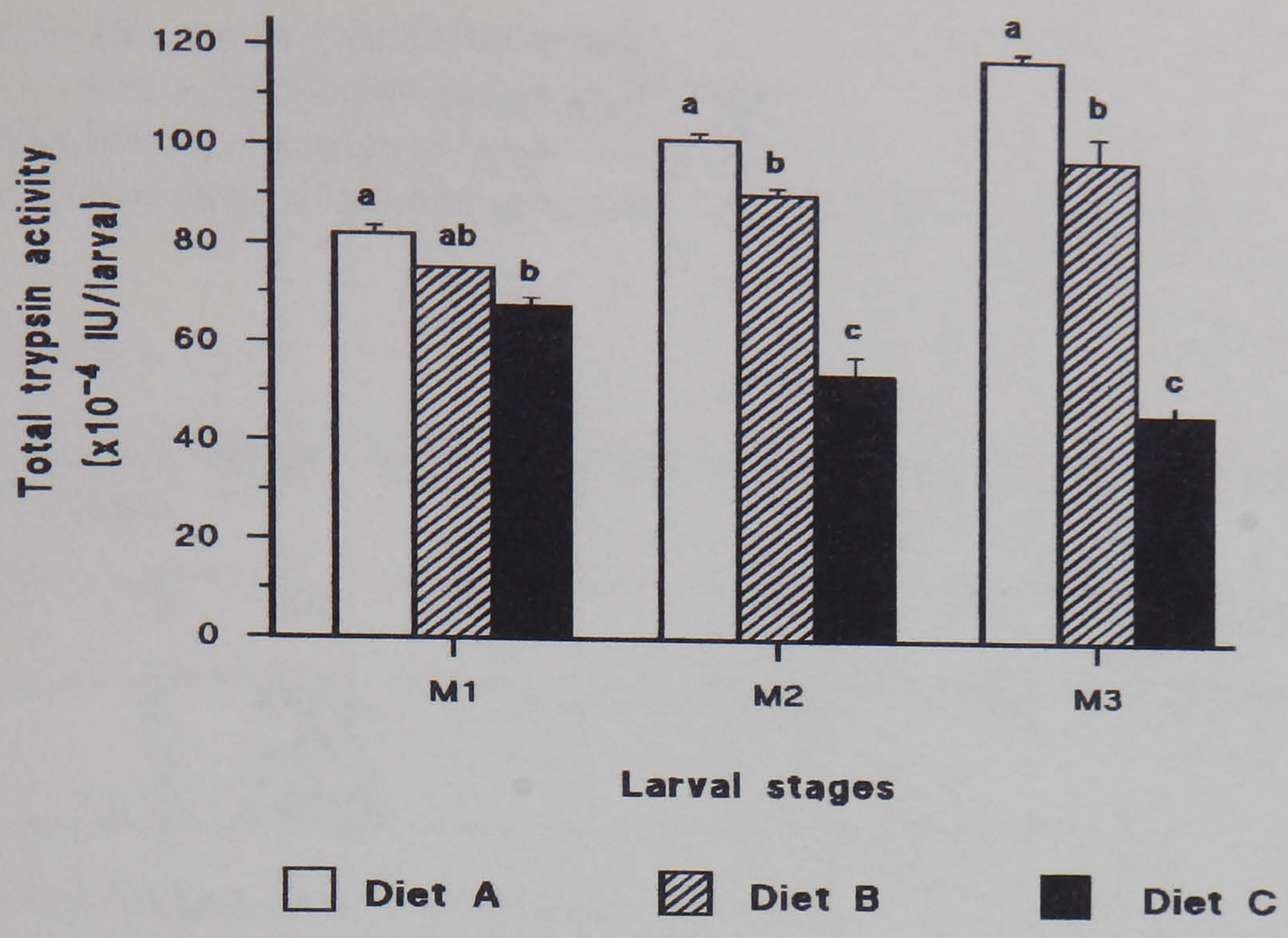


Figure 5. Total trypsin activity of larvae reared on Diet A (MED), Diet B (MED+one *Artemia* ml^{-1}), and Diet C (MED + five *Artemia* ml^{-1}) from M1 to PL1 stages. Values are means ± s.d., n=2 for each treatment, n= at least three for each assay. With each larval stage, treatments marked with different superscripts are significantly different ($P<0.05$).

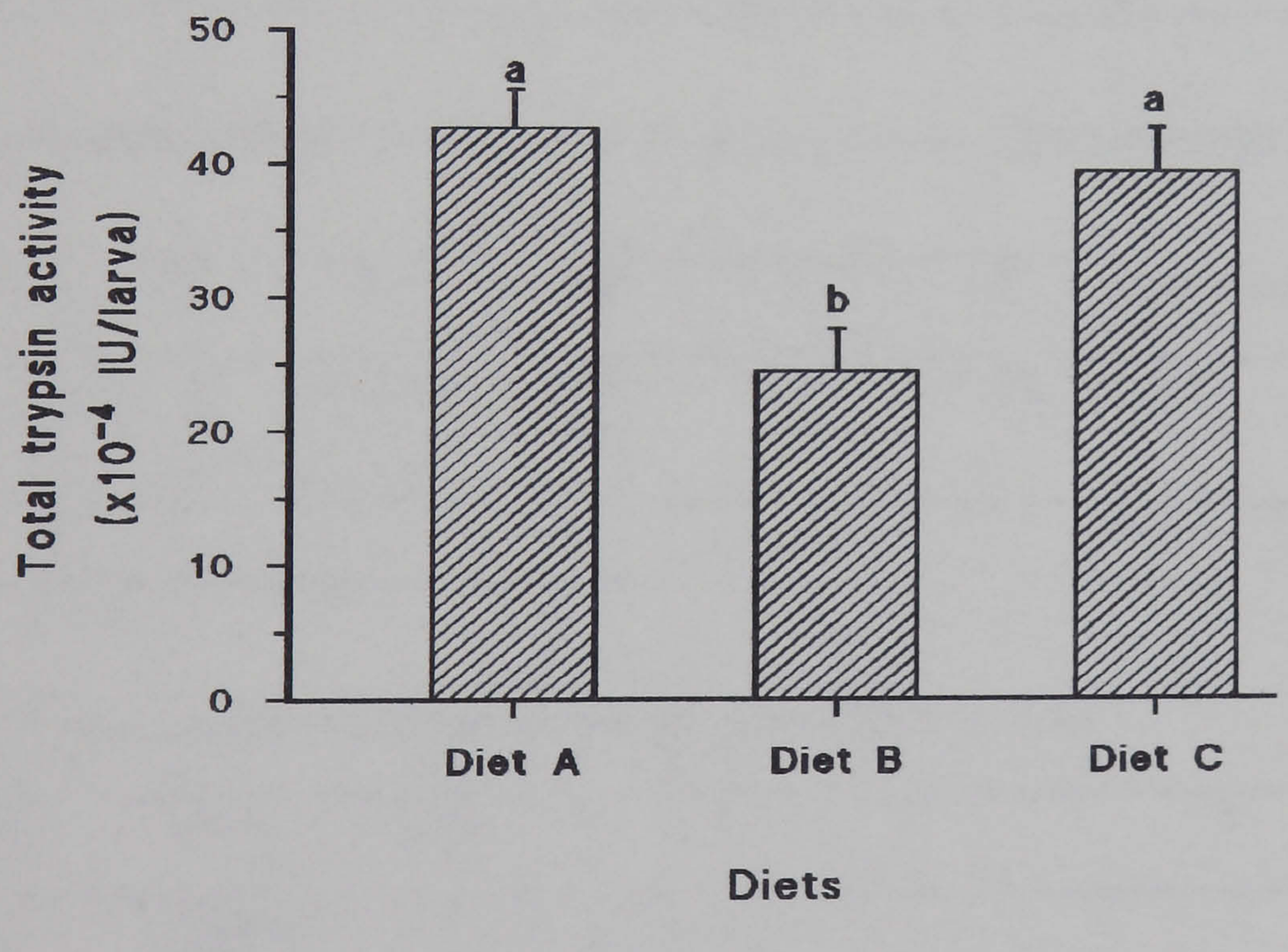


Figure 6. Total trypsin activity of the larvae reared on live algae, *R. reticulata* (Diet A), microencapsulated diet (Diet B), and algae incorporated microencapsulated diet (Diet C) from PZ1 to PZ2 stages. Each value is a mean ± s.d. (n=2). Treatments marked with different superscripts are significantly different ($P<0.05$).

(Ns) Indicates a non-significant result.
(***) Indicates a significant result at P≤ 0.001.
(**) Indicates a significant result at P≤0.01.
(*) Denotes pairs of groups are significantly different at p≤0.05.

Experiment 1

Table 2a. One-way ANOVA on larval survival of *P. indicus* on various diets from PZ1 to PZ3 stage.

SOURCE	DF	SS	MS	F	P	Significant
Diets	4	658.65	164.66	23.95	0.002	**
Error	5	34.37	6.87			
Total	9	693.03				

Bartlett's test for homogeneity of variances
F= 3.42, P= 0.16

Table 2b. Tukey's pairwise comparison test on survival at PZ3 stage.

Survival	Diets	Code	1	2	3	4	5
78.50	Control	1					
66.75	MED-8	2	*				
71.25	MED-6	3	Ns	Ns			
59.25	MED-4	4	*	Ns	*		
56.00	MED-10	5	*	*	*	Ns	

Table 3a. One-way ANOVA on survival of *P. indicus* fed on various diets until PL1 stage.

SOURCE	DF	SS	MS	F	P	Significant
Diets	4	1062.2	265.5	25.66	0.002	**
Error	5	51.7	10.4			
Total	9	1113.9				

Bartlett's test for homogeneity of variances
F= 3.93, P= 0.14

Table 3b. Tukey's pairwise comparison test on survival at PL1 stage.

Survival	Diets	Code	1	2	3	4	5
68.50	Control	1					
58.25	MED-8	2	Ns				
53.00	MED-6	3	*	Ns			
43.25	MED-4	4	*	*	Ns		
40.00	MED-10	5	*	*	*	Ns	

Table 4a. One-way ANOVA on larval growth of *P. indicus* fed various diets from PZ1 to PZ3 stage.

SOURCE	DF	SS	MS	F	P	Significant
Diet	4	2.6526	0.6632	39.62	0.000	***
Error	95	1.5899	0.0167			
Total	99	4.2426				

Bartlett's test for homogeneity of variances
F= 4.02, P= 0.13

Table 4b. Scheffé's pairwise comparison test on larval growth at PZ3 stage.

Growth	Diets	Code	1	2	3	4	5
3.438	Control	1					
3.094	MED-8	2	*				
3.030	MED-6	3	*	Ns			
2.992	MED-4	4	*	Ns	Ns		
3.047	MED-10	5	*	Ns	Ns	Ns	

Table 5a. One-way ANOVA on growth of larvae at PL1 stage.

SOURCE	DF	SS	MS	F	P	Significant
Diet	4	35.012	8.753	82.48	0.000	***
Error	100	10.612	0.106			
Total	104	45.624				

Bartlett's test for homogeneity of variances
P= 0.98, P= 0.15

Table 5b. Scheffé's pairwise comparison test on larval growth at PL1 stage.

Growth	Diets	Code	1	2	3	4	5
6.846	Control	1					
5.522	MED-8	2	*				
5.334	MED-6	3	*	Ns			
5.206	MED-4	4	*	*	Ns		
5.721	MED-10	5	*	Ns	*	*	

Experiment 2

Table 6. Body dry weight (µg) of *P. indicus* larvae fed live algae and *Artemia* (M1 onwards) from PZ1 to PL1 stage.

Larval body dry weight (µg)						
PZ1	PZ2	PZ3	M1	M2	M3	PL1
4.167 ± 0.034	8.017 ± 1.337	11.830 ± 0.806	17.455± 0.898	37.500± 3.068	71.668± 3.773	80.835± 5.890

Table 7. Trypsin data for Figure 1. Each value is a mean ± s.d. n=2 for each stage and n=3 for each assay.

Stages	Total trypsin activity (x 10^{-4} IU/larva)	Specific trypsin activity (x 10^{-5} IU/ µg DW)
PZ1	4.596 ± 0.540	9.253
PZ2	18.412 ± 0.765	22.967
PZ3	60.813 ± 3.969	51.408
M1	129.000 ± 2.674	73.904
M2	121.256 ± 4.925	32.335
M3	62.466 ± 2.478	8.716
PL1	53.801 ± 1.717	6.656

Experiment 3

Table 8a. Two-way ANOVA with days as a covariate on survival of *P. indicus* larvae fed various diets from PZ1 to PZ3/M1 stages.

	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	4	5310.13	16.37	4.09	0.30	0.874	
Days	1	1394.45	1394.45	1394.45	100.93	0.000	
Repl(Diets)	5	27.17	32.63	6.53	0.47	0.789	
Diets*Days	4	873.67	873.67	218.42	15.81	0.000	***
Repl*Days(Diets)	5	61.38	61.38	12.27	0.89	0.524	
Error	10	138.17	138.17	13.82			
Total	29	7804.97					

Table 8b. Comparison of mortality rates of *P. indicus* larvae fed on diets (described in Figure 2) during herbivoral stages (PZ1 to PZ3/M1).

	Mortality rates (% day^{-1})	Coeff.	Stdev.	t-value	P	Significant
Constant		98.167	1.796	54.67	0.000	
Average Slope		-8.35	0.8312	-10.05	0.000	
Diet A	1.063	6.225	1.662	3.74	0.004	**
Diet B	8.750	-9.150	1.662	-5.50	0.000	***
Diet C	1.813	4.725	1.662	2.84	0.017	*
Diet D	1.625	5.1	1.662	1.14	0.280	Ns
Diet E	7.625	-6.900	1.662	-4.15	0.002	**

Table 9a. Two-way ANOVA with days as a covariate on survival of *P. indicus* larvae fed on various diets between M1 and PL stages.

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	4	12812.31	1056.83	264.21	57.96	0.000	
Days	1	686.35	686.35	686.35	150.56	0.000	
Repl(Diets)	5	73.59	116.69	23.34	5.12	0.003	
Diets*Days	4	126.27	126.27	31.57	6.92	0.001	***
Repl*Days(Diets)	5	105.89	105.89	21.18	4.65	0.006	
Error	20	91.18	91.18	4.56			
Total	39	13895.59					

Table 9b. Comparison of mortality rates of *P. indicus* larvae fed on various diets (described in Figure 2) between M1 and PL stages.

	Mortality rate (% day^{-1})	Coeff.	Stdev.	t-value	P	Significant
Constant		84.235	1.4	60.16	0.000	
Average slope		-3.7050	0.3020	-12.27	0.000	
Diet A	1.625	0.4550	0.6039	0.75	0.460	Ns
Diet B	1.663	0.3800	0.6039	0.63	0.536	Ns
Diet C	3.313	-2.9200	0.6039	-4.84	0.000	***
Diet D	1.778	0.1550	0.6039	0.26	0.800	Ns
Diet E	0.888	1.9300	0.6039	3.2	0.003	**

Table 10a. Two-way ANOVA with days as a covariate on total length of *P. indicus* larvae fed on various diets during herbivoral stages (PZ1 to PZ3/M1).

Source	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diets	4	76.706	0.078	0.020	0.28	0.890	
Days	1	249.846	249.261	249.261	3564.16	0.000	
Repl(Days)	5	0.062	0.127	0.025	0.36	0.874	
Diets*Days	4	12.797	12.782	3.196	45.69	0.000	***
Repl*Days(Diets)	5	0.140	0.140	0.028	0.40	0.849	
Error	281	19.652	19.652	0.070			
Total	300	359.232					

Table 10b. Comparison of growth rates of *P. indicus* larvae fed on various diets (described in Figure 2) during herbivoral stages (PZ1 to PZ3/M1).

	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Significant
Constant		1.03187	0.03997	25.82	0.000	
Average Slope		1.11359	0.01865	59.70	0.000	
Diet A	0.787	0.46041	0.03738	12.32	0.000	***
Diet B	0.424	-0.26607	0.03710	-7.17	0.000	***
Diet C	0.552	-0.00977	0.03705	-0.26	0.792	Ns
Diet D	0.561	0.00690	0.03705	0.19	0.852	Ns
Diet E	0.462	-0.19146	0.03795	-5.05	0.000	***

Table 11a. Two-way ANOVA with days as a covariate on total length of *P. indicus* larvae fed on various diets between M1 and PL stages.

	DF	Seq SS	Adj SS	Adj MS	F	P	Significant
Diet	4	461.909	12.508	3.127	26.69	0.000	
Days	1	389.116	385.024	385.024	3286.11	0.000	
Repl(Diets)	5	0.519	0.430	0.086	0.73	0.593	
Diets*Days	4	49.061	48.894	12.223	104.33	0.000	***
Repl*Days(Diets)	5	0.636	0.636	0.127	1.09	0.368	
Error	376	44.055	44.055	0.117			
Total	395	945.296					

Table 11b. Comparison of growth rates of *P. indicus* larvae fed on various (described in Figure 2) diets during mysis stages (M1-PL1).

	Growth rate (mm day^{-1})	Coeff.	Stdev.	t-value	P	Significant
Constant		0.61160	0.07223	22.31	0.000	
Average Slope		0.89876	0.01568	57.32	0.000	
Diet A	0.666	0.43360	0.03108	13.95	0.000	***
Diet B	0.293	-0.31365	0.03081	-10.18	0.000	***
Diet C	0.237	-0.42464	0.03200	-13.27	0.000	***
Diet D	0.518	0.13620	0.03108	4.38	0.000	***
Diet E	0.534	0.16848	0.03180	5.30	0.000	***

Table 12a. Two-way ANOVA on total trypsin activity of *P. indicus* larvae fed on various diets from PZ1 to M1 stage.

Source	DF	SS	MS	F	P	Significant
Diets	2	1392.7	696.3	101.70	0.000	***
Stages	3	20464.2	6821.4	996.25	0.000	***
Diets*Stages	6	346.6	57.8	8.44	0.001	***
Error	13	82.2	6.8			
Total	17	22285.6				

Table 12b. Tukey's test on trypsin activity of larvae fed on diets (described in Figure 3) between PZ1 and M1 stage.

Diets	trypsin	Codes	1	2	3
Diet A	54.757	1			
Diet B	36.120	2	*		
Diet C	44.659	3	*	*	

Table 13. Total trypsin activity of *P. indicus* fed on diets (described in Figure 3). Each value is a mean ± s.d. (n=2). Treatments with same superscripts are not significantly different from each other (P>0.05).

	Total trypsin Activity ($x10^{-4}$ IU $larva^{-1}$)		
Stages	Diet A	Diet B	Diet C
PZ1 (at Hatch)	6.907 ± 0.839	6.907±0.839	6.907±0.839
PZ1(24h fed)	16.279 ± 1.462^{a}	7.396 ± 1.667^{b}	11.974 ± 0.596^{ab}
PZ2	33.883 ± 0.569^{a}	21.025 ± 1.713^{b}	26.767 ± 2.664^{ab}
PZ3	62.532 ± 4.118^{a}	40.190 ± 4.092^{b}	54.924 ± 1.940^{ab}
M1	106.335 ± 4.223^{a}	75.868 ± 2.663^{b}	84.972 ± 2.073^{b}

Table 14a. Two-way ANOVA on specific trypsin activity of *P. indicus* fed on various diets from PZ1 to M1 stage.

Source	DF	SS	MS	F	P	Significant
Diets	2	482.23	241.11	29.50	0.000	***
Stages	3	3381.33	1127.11	137.92	0.000	***
Diets*Stages	6	196.99	32.83	4.02	0.019	*
Error	12	98.07	8.17			
Total	23	4158.61				

Table 14b. Tukey's pairwise comparison test on specific trypsin activity of larvae fed on three feeding regimes (described in Figure 3).

Diets	trypsin	Codes	1	2	3
Diet A	47.659	1			
Diet B	37.131	2	*		
Diet C	45.096	3	Ns	*	

Table 15. Specific trypsin activity of *P. indicus* larvae fed on diets as described in Figure 3 from PZ1 to M1. Values are means ± s.d., n=2 for each treatments and n=3 for each assay. Diets with same superscripts are not significantly different (P>0.05).

Larval Stages	Specific trypsin activity ($x\ 10^{-5}$ IU μg^{-1} DW)		
	Diet A	Diet B	Diet C
PZ1 (24 h fed)	32.72 ± 1.873^{a}	16.26 ± 2.248^{b}	25.95 ± 1.294^{a}
PZ2	45.44 ± 0.076^{a}	34.94 ± 0.721^{b}	43.27 ± 1.427^{a}
PZ3	49.89 ± 1.859^{b}	44.79 ± 1.739^{b}	57.44 ± 2.029^{a}
M1	62.59 ± 0.248^{a}	52.53 ± 0.189^{b}	53.73 ± 0.131^{b}

Table 16. Dry weight (µg) of *P. indicus* fed various diets (as in Figure 3) from PZ1 to M1 stage.

Stages	Diet A	Diet B	Diet C
PZ1 (24h fed)	4.976 ± 0.412	4.548 ± 0.561	4.613 ± 0.125
PZ2	7.456 ± 1.220	6.017 ± 1.050	6.187 ± 0.478
PZ3	12.532 ± 0.925	8.972 ± 2.176	9.562 ± 0.723
M1	16.987 ± 2.231	14.430 ± 3.185	15.813 ± 1.140

Table 17a. Two-way ANOVA on total trypsin activity during mysis stages of *P. indicus* fed on various diets until PL1.

Source	DF	SS	MS	F	P	Significant
Diets	4	12835.7	3208.9	219.74	0.000	***
Stages	2	3882.3	1941.2	132.93	0.000	***
Diets*Stages	8	1689.6	211.2	14.46	0.000	***
Error	15	219.1	14.6			
Total	29	18626.7				

Table 17b. Tukey's test for trypsin activity of *P. indicus* larvae (described in Figure 3) from M2 to PL1.

Diets	Trypsin	code	1	2	3	4	5
Diet A	64.387	1					
Diet B	89.859	2	*				
Diet C	94.525	3	*	Ns			
Diet D	52.725	4	*	*	*		
Diet E	41.884	5	*	*	*	*	

Table 18. Total trypsin activity of *P. indicus* fed on diets described in Figure 3 between M1 and PL1 stages. Each value is a mean ± s.d. (n=2). Values with same superscripts are not significantly different from each other (P>0.05).

	Total trypsin activity ($x10^{-4}$ IU $larva^{-1}$)				
Stages	Diet A	Diet B	Diet C	Diet D	Diet E
M2	83.349 ± 5.048^{a}	91.990 ± 5.642^{a}	87.293 ± 2.381^{a}	65.620 ± 2.137^{b}	52.778 ± 1.704^{c}
M3	60.612 ± 3.574^{b}	116.455 ± 3.112^{a}	106.703 ± 3.611^{a}	58.746 ± 2.217^{bc}	43.233 ± 4.290^{c}
PL1	49.199 ± 1.356^{b}	75.130 ± 0.169^{a}	75.580 ± 2.976^{a}	33.809 ± 1.853^{c}	29.641 ± 1.897^{c}

Experiment 4

Table 19a. One-way ANOVA on larval survival (%) of *P. indicus* on various diets at PL1 stage.

SOURCE	DF	SS	MS	F	P	Significant
Diets	2	1702.50	851.25	95.42	0.002	**
Error	3	26.76	8.92			
Total	5	1729.27				

Bartlett's test for homogeneity of variances
F= 0.356, P= 0.42

Table 19b. Tukey's test on larval survival (%) of *P. indicus* on various diets (described in Figure 4) at PL1 stage.

Diets	Trypsin	code	1	2	3
Diet A	30.355	1			
Diet B	54.285	2	*		
Diet C	71.430	3	*	*	

Table 20a. One-way ANOVA on larval growth (mm TL) of *P. indicus* on various diets at PL1 stage.

SOURCE	DF	SS	MS	F	P	Signifi-cant
Diets	2	3.9638	1.9819	23.36	0.000	***
Error	58	4.9212	0.0848			
Total	60	8.8850				

Bartlett's test for homogeneity of variances
F= 4.558, P= 0.40

Table 20b. Scheffé's pairwise comparison test on larval growth (mm TL) of *P. indicus* on various diets (described in Figure 4) at PL1 stage.

Diets	Trypsin	code	1	2	3
Diet A	5.338	1			
Diet B	5.735	2	*		
Diet C	5.941	3	*	Ns	

Table 21. Survival, growth (dry weight) and biomass (number of larvae x µg DW) of *P. indicus* larvae fed various diets from PZ1 to PZ3/M1 stages.

Diet	Survival (%)	Larval stages (%)	Dry weight (µg)		Biomass at stages		Total biomass (µg)
			PZ3	M1	PZ3	M1	
Mixed algae	95.75 (day 4)	5% PZ3; 95% M1	12.532	16.987	120	3090	3210
MED+algae	91.00 (day 6)	100% M1		15.813		2878	2878
MED	46.75 (day 6)	67% PZ3; 33% M1	8.972	14.430	562	445	1007

DISCUSSION

Conventional feeding regimes of penaeid larvae are based on a combination of live feeds, with microalgae at protozoeal stages and *Artemia* at mysis and early postlarval stages. Galgani and Aquacop (1988) report that an algal concentration of 30-40 cells μl^{-1} is adequate for rearing *P. indicus* larvae during protozoeal stages. However, in Chapter 1, the larvae of this species were cultured from PZ1 to PL1 within only 6-7 days with over 90 % survival in water at 25 ppt salinity (S) on 60-70 cells μl^{-1} of live algae. In Chapter 1 superior growth and survival was obtained from mixed algae during herbivorous stages (*S. costatum* and *T. chuii*) rather than a single algae species. Therefore, in present work, control larvae were fed on a mixed algal diet throughout all stages and plus five *Artemia* ml^{-1} at mysis stages to ensure sufficient quality and quantity of food during the culture. *P. indicus* larvae were found to survive and grow better at lower salinities (Chapter 1), hence the salinity level of present culture water was maintained at 25 ppt.

Present results indicate that feeding level of MED for *P. indicus* is similar to that previously reported for other penaeid species such as *P. monodon* (Kurmaly et al., 1989a), and *P. japonicus* (Le Vay et al., 1993). The best feeding level appears to be 6-8 mg l^{-1} with four distribution times a day for the culture of this species during protozoeal stages. Even this level of MED as a sole diet retarded development of the larvae by 1.5-2 days in comparison to live control diet from PZ1 to PZ3/M1 stages. Higher feeding rates (10 mg l^{-1}) fouled the larvae and caused high mortalities (Jones et al., 1979a). Attempts to rear *P. indicus* larvae on artificial diets without antibiotic failed, confirming the findings of Galgani and Aquacop (1988). Complete replacement of live diets (both algae and *Artemia*) with MED resulted in 37 % survival and 5.42 mm final total length at PL1 compared to 88 % and 6.75 mm total length of the live control (Figures 2a, b). However, pre-

sent results also indicate that *P. indicus* larvae can be reared in the absence of an animal prey from PZ1 to PL1/2 with a 66 % survival and a total length of 5.83 mm on the MED plus 15 cells μl^{-1} of frozen mixed algae (Figures 2a, b).

Specific trypsin (per µg dry weight) and total trypsin activity (per organism) of *P. indicus* larvae fed live diets during larval development were found to be similar to those of *P. monodon* (Kamarudin, 1992; Kumlu et al., 1992; Jones et al., 1993), *P setiferus* (Lovett and Felder, 1990b) and *P. japonicus* (Galgani and Benyamin, 1985; Le Vay et al., 1993). Specific trypsin activity on the live diets is low during PZ1 stage (9.25×10^{-5} IU μg^{-1} DW) rising sharply during herbivorous stages (PZ1-PZ3) reaching a peak at M1 (74×10^{-5} IU μg^{-1} DW) and falling during subsequent stages until PL1 stage (6.66×10^{-5} μg^{-1} DW) (see Figure 1). 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 post-larval stages. When stage PZ1 larvae are fed on microencapsulated diets, their trypsin-like enzyme response is significantly lower than when fed either MED with algal co-feed or live diets (see Figure 3) and (Le Vay et al., 1993). Slower response of digestive enzymes 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 (Figure 2a). Once the larvae pass this critical stage, their digestive capability appears to be better able to respond to MED.

Present results suggest the role of algae as a gut enzyme stimulant for *P. indicus* larvae and agree with the results obtained from other penaeid species (Kamarudin, 1992; Kumlu et al., 1992; Le Vay et al., 1993). It seems that the level of larval trypsin activity responds positively to the presence of an algal co-feed particularly during herbivorous stages. Since the larvae appear incapable of responding in the same way to artificial diets, addition

of algae is especially important during the first days of culture. This is confirmed by the findings in Chapter 4 where the provision of live algae for only 24 h as a co-feed (15 cells μl^{-1}) with live nematodes (*Panagrellus redivivus*) significantly elevated larval trypsin activity and accelerated the growth and survival of *P. indicus* larvae from PZ1 to PZ3 stage in comparison to the nematode only fed larvae.

Present results confirm that enhanced tryptic activity during protozoeal stages correlates with improved survival and growth (Kamarudin, 1992; Jones et al., 1993). Addition of 15 cells μl^{-1} of frozen algae increased survival from 47-58 (MED only) to 85-91 % (MED plus alga), with a total length increase from 3.66 mm to 4.31 mm at stage PZ3 (Figures 2a, b). Larval development was, however, retarded 1-2 days with final growth still inferior to that on mixed live algae at M1.

It may be argued that the addition of 10-15 cells μl^{-1} to the MED might cause higher larval trypsin activity as a result of a higher food concentration or contribute extra nutrients resulting in improved growth and survival of the penaeid larva. However, present experiments (Figure 5) show that an increase in food concentration, at least in the mysis stages, produces a drop in digestive enzyme activity. In addition, when the algae were incorporated into microcapsules and fed at the same concentration as controls (Figure 6) elevation of enzyme levels still occurred. Although it is difficult to eliminate the possibility of micronutrients contributing to increased survival and growth, *Rhinomonas reticulata* used in the present experiments (Figure 6) has been demonstrated to be unsuitable for the culture of *P. indicus* (see Chapter 1). When this alga is fed either alone or in mixed culture with other algae, it produces poor growth and survival. In present work when it is incorporated into capsules, fed live or frozen at low densities with MED, it enhances trypsin level. Similarly, Rodríguez et al., (1994) found that

Chaetoceros gracilis, containing only 7 % protein, produces high enzyme activity in early larval *P. japonicus*.

Table 21 shows that biomass (number of larvae x larval body dry weight = DW) of larvae reared on MED, and MED plus 15 cells μl^{-1} frozen algae, at PZ3/M1 stages (Experiment 3), are 1007 µg and 2878 µg DW respectively. When the contribution of the 15 cells μl^{-1} algal co-feed (803 µg DW), (1/4 of the live algal control treatment), is added to the biomass produced by the MED 1007+803 =1810 µg DW), there is an excess larval biomass (1068 µg DW) produced by MED plus 15 cells μl^{-1} frozen algae. The extra larval biomass obtained by the addition of 15 cells μl^{-1} of frozen algae cannot be accounted simply by dry weight input of this algae alone. Hence it is suggested that the excess biomass (1068 µg DW) must come from enhanced digestion of the artificial diet due to enzymatic activity generated by the algal compounds (see also Rodríguez et al., 1994).

In contrast to the situation in protozoeal stages, algae do not appear to provide the same enhancement of digestive enzyme activity in *P. indicus* mysis larval stages. Trypsin activity in mysis stage larvae declines after the larvae commence feeding upon *Artemia* nauplii (Figures 3, 5). This decline has been attributed to the easily digestible nature of live prey (Jones et al., 1993; Le Vay et al., 1993) rather than degeneration of the AMD (Abubakr and Jones, 1992). Although exogenous enzymes from live prey are important for first feeding fish larvae (Lauff and Hofer, 1984; Munilla-Moran et al., 1990), their contribution is insignificant in penaeid mysis and late *Macrobrachium* larval digestion (Kamarudin, 1992; Jones et al., 1993). When *P. indicus* mysis larvae are reared on MED without *Artemia* nauplii, trypsin activity remains significantly elevated above treatments receiving these diets together with *Artemia* (Figures 3, 5). These results confirm the view that the larvae secrete high levels of digestive enzymes when substrate nu-

trients such as protein are scarce or less digestible (Jones et al., 1993; Le Vay et al., 1993).

The present study is encouraging in that algae incorporated into microencapsulated diets, which promoted better survival and growth rates in penaeid larvae, have been successfully retained in the diet with this microencapsulation technique (Figure 6). These *R. reticulata* containing microcapsules induce equal trypsin activity to that of live algae and a significantly higher level of trypsin than capsules without algal inclusion. Incorporation of microalgae into microcapsules should improve survival and growth of herbivorous stage penaeid larvae, whereas incorporation of pre-digested dietary ingredients may overcome the poor digestibility of diets for the mysis larvae.

CHAPTER 6

FEEDING AND DIGESTION IN THE CARIDEAN SHRIMP LARVAE *Palaemon elegans* AND *Macrobrachium rosenbergii* ON LIVE AND ARTIFICIAL DIETS.

This chapter has contributed to the following publication:

Title: Feeding and digestion in the caridean shrimp larvae *Palaemon elegans* Rathke and *Macrobrachium rosenbergii* De Man (Crustacea: Palaemonidae) on live and artificial diets.

Authors: Kumlu, M. and Jones, D. A.

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INTRODUCTION

Complete replacement of natural diets in larval fish and prawn culture has long been the main goal of nutritionists in aquaculture. To achieve this aim, an artificial diet must satisfy some physical (appropriate size, shape, texture, stability, specific gravity) and chemical features (taste, balanced nutritional contents) and also must be designed according to morphological and physiological structure of the digestive system of an organism.

Several authors have reported very good growth and survival results for partial or complete replacements of live diets with microencapsulated diets (MED) (Jones et al., 1987; Kurmaly et al., 1989a; Amjad et al., 1992) and with microparticulated diets (Kanazawa et al., 1982; Galgani and Aquacop, 1988; Ottogali, 1991) for rearing penaeid larvae, both in the laboratory and in hatcheries. The capability of penaeid larvae to feed solely on artificial diets has been attributed to their short gastroevacuation time and high digestive enzyme activity during their early stages (Jones and Kurmaly, 1987; Abubakr and Jones, 1992).

In contrast, there has been no report of any successful complete replacement of live diets for caridean larvae (Jones et al., 1975; Deru, 1990). These larvae hatch as zoea and usually are fed exclusively on live *Artemia* during early stages with artificial supplements during later stages (Ling, 1969a, b; Rochanaburanon and Williamson, 1976). Villamar and Brusca (1987) reported that *Crangon nigricauda* larvae were reared successfully until megalopa stages on *Chaetoceros gracilis*, whereas larvae fed *Artemia* failed to reach that stage. Ling (1969a, b) was the first to achieve culture of *Macrobrachium rosenbergii* larvae on live zooplankton (i.e. rotifers, copepods, and insect larvae) and on a combination of live and artificial diets (i.e. chopped fish, shellfish, steamed egg custard, and fish eggs). Several authors have tried to completely replace live *Artemia* nauplii, which may

constitute 60% of the cost of hatchery operations (Menasveta et al., 1984), with other live zooplankton (Lovett and Felder, 1988) or artificial diets (Jones et al., 1975; Sick and Beaty, 1975; Murai and Andrews, 1978). Promising results were reported by Deru (1990), who achieved complete replacement of live *Artemia* in culturing *M. rosenbergii* from stage Z6 to Z11 with over 80 % survival using a Frippak CD3 microencapsulated diet designed for penaeid larvae. This diet, however, could not sustain development of the early larvae further than stage Z4.

These studies with caridean larvae suggest that failures to substitute for live diets are not due to major deficiencies in nutritional content of the diets, but are more likely to be due to the undeveloped digestive system of these species during early larval stages. Extensive studies on the gut structure of *M. rosenbergii* (Deru, 1990) and *Palaemon elegans* (Abubakr, 1991) reveal that the early larvae lack the anterior midgut diverticulae which form the main site for digestive enzyme production during early stages in penaeids. The hepatopancreas is also relatively small during early zoeal stages, but the volume of this organ increases drastically after stage Z5-6. Therefore, it might be expected that a considerable increase in digestive enzyme activity levels may coincide with the expansion of hepatopancreas after stage Z5, when they are able to survive on artificial diets (Deru, 1990).

If an appropriate artificial diet is to be developed for these caridean carnivorous larvae, their digestive physiology, morphology and feeding behaviour must be fully understood. To date, no information is available on gastroevacuation time, feeding behaviour and digestive enzyme response of *P. elegans* in relation to diets. Kamarudin et al., (1994) studied larval digestive enzymes in *M. rosenbergii* and concluded that the larvae should have enough digestive enzymes to digest artificial diets even during early larval stages. However, present studies indicate that carnivorous decapod larvae

appear to show low proteolytic enzyme production in comparison to herbivorous penaeid larvae during protozoeal stages. The objective of this study was, therefore, to further investigate aspects of digestive physiology, such as trypsin activity, gastroevacuation time and feeding behaviour, of caridean larvae *P. elegans* and *M. rosenbergii,* which have similarities in larval gut morphology, feeding behaviour and larval development using live and artificial diets.

MATERIALS AND METHODS

Survival and Growth

Palaemon elegans

Gravid female (50-55 individuals) *Palaemon elegans* were collected by hand net from shallow intertidal pools on Church Island, Menai Bridge. They were placed in an aquarium (1 x 0.7 x 0.7 m) supplied by water recirculated through a gravel filter. The prawns were fed fresh mussel, *Mytilus edulis*, daily and occasionally squid. Water temperature of the aquaria was kept at 25 °C by a thermostatically controlled heater. The newly hatched larvae were rinsed with UV-irradiated and filtered (0.2 µm) sea water and stocked at a density of 50 larvae l^{-1} into 2-litre round-bottom glass flasks. These flasks were previously washed thoroughly with hot water and sodium hypochlorite solution. After stocking the larvae, the flasks were placed in a thermostatically controlled water bath at 25 °C and each flask was aerated via a silicon rubber tube and a glass rod at the tip. *Artemia* cysts and all artificial diets used during the present experiments were obtained from INVE AQUACULTURE, Belgium. These diets which contain 49-57 % protein, 7.5-27 % carbohydrate, 13-23.5 % lipid together with mineral and vitamin mixes (Kurmaly et al., 1989a, Amjad et al., 1992) and have same energy levels as live feeds (Kurmaly et al., 1989b) have been used successfully as a complete replacement for live feeds for penaeid lar-

val culture (Jones et al., 1987; Le Vay et al., 1993). Control larvae were fed newly hatched *Artemia salina* nauplii at 15 ml^{-1} through all larval stages. A Frippak microgranulated diet (PL+150 brand) at 10 mg l^{-1} (100-200 µm) was fed to Z1 stage larvae to determine whether it would sustain larval survival. Some Z1 stage larvae were starved as a control treatment.

In a separate experiment, Z4-5 stage larvae previously fed *Artemia* in two 5-l round-bottom glass flasks were again stocked in 2-l experimental flasks and were starved, fed *Artemia* (15 ml^{-1}) or a Frippak microgranulated diet (PL+300 brand) at 14 mg l^{-1} (200-400 µm) until PL1 stage.

The artificial diets were hydrated daily and distributed into the flasks four times a day (8.00, 14.00, 18.00, 24.00 h). Larval survival and growth were assessed from two replicates for each treatment. Every other day, the water in the flasks was renewed and all the larvae were counted. A sample of 10-13 larvae were measured from the tip of the rostrum to the end of telson under a binocular microscope and staged according to Fincham (1977). Growth and survival rates of the larvae were compared using General Linear Model (GLM) in Minitab statistical software.

Macrobrachium rosenbergii

A gravid female *M. rosenbergii* was placed in a 100-litre freshwater tank at 28-29 °C. Newly hatched larvae were siphoned out, rinsed with clean water and 50 larvae l^{-1} were stocked in 2-litre round-bottom glass flasks with water at 12 ppt salinity (S) to assess their survival and growth. Rearing water was obtained by mixing filtered (0.2 µm) and UV-treated sea water with distilled water. Larval culture was conducted at 29-30 °C by keeping the flasks in a thermostatically controlled water bath. As early larvae cannot survive on artificial diets (Deru, 1990), this experiment was performed with later stage (Z5-6) *M. rosenbergii* larvae and continued until

metamorphosis. Control larvae were fed *Artemia salina* nauplii at 15 ml^{-1}. A Frippak microencapsulated diet (CD3 type) designed for penaeid mysis stages was sieved through 140-µm mesh, and 12 mg l^{-1} of the remaining larger particles were used to feed the larvae from Z5-6 (12 day-old) to Z10. After this stage, since microencapsulated diet with larger particles is not currently available, 14 mg l^{-1} of Frippak microgranulated diet (200-400 µ) was used until the PL1 stage. Complete water change of the flasks was carried out every other day when larval survival was assessed and total length of 10-12 larvae was measured from the tip of the rostrum to the end of telson and staged according to Ling (1969a), and New and Singholka (1985). Larval growth rates between day 20 and day 28 were compared using GLM.

Trypsin response of *M. rosenbergii* larvae starved, fed artificial diets and *Artemia* was also investigated. For this purpose, stock larvae were reared in a 50-l tank and fed 10-15 newly hatched *Artemia* nauplii ml^{-1}. At early (Z2 and Z4) and late stages (Z6 and Z9), these larvae were transferred into 2-l experimental flasks and were starved, fed artificial diets (INVE AQUACULTURE, Belgium) or *Artemia* (15 ml^{-1}) for a period of 24 h before they were sampled for trypsin analysis. The following artificial diets were used;

(A) 10 mg l^{-1} of Frippak CD3 diet (100 µm) for Z2 and Z4 stages,
(B) 12 mg l^{-1} of Frippak PL+150 diet (100-200 µ) for Z6 stage,
(C) 16 mg l^{-1} of Frippak PL+300 (200-400 µ) diet for Z9 stage.

At the end of each trial, 30-50 larvae starved, fed on formulated diets, or *Artemia* were sampled in two replicates for the enzyme assays. Results were compared using one-way ANOVA and Tukey's pairwise comparison test.

Feeding Behaviour and Gastroevacuation time

The larvae of both species were observed under a binocular microscope in petri dishes to assess the gastroevacuation time and feeding behaviour during early and late stages. For gastroevacuation time (GET), *Artemia* nauplii with guts full of 30 µm charcoal particles were fed to the larvae which were already feeding normally on *Artemia*. The GET was estimated as the difference in time between the appearance of the labelled food in the fore-gut and the complete clearance of the hind-gut. *Artemia* predation rate by *M. rosenbergii* larvae was also determined by counting the remaining *Artemia* after 24 h in three replicate flasks (500 ml) containing larvae, and comparing with control flasks without larvae. In addition, predation by larvae on *Artemia* and formulated diets was also continuously observed for 3-4 h under the microscope.

Samples for trypsin assays of *P. elegans* were obtained from the larvae that were reared in three 5-l round bottom flasks and fed on 15 *Artemia* nauplii ml^{-1}. The same amount of *Artemia* was used for *M. rosenbergii* larvae reared in a 10-litre round bottom glass flask. 50 % of the water of these large flasks was renewed daily. Various numbers of larvae (50 for early and 25 for late stages), starved, fed *Artemia* or artificial diets, were transferred into 1-ml microtest tubes and stored at -70 °C in a ultracold freezer before the enzyme assays were conducted. Whole homogenates were used in the enzyme assays as explained in General Material and Methods (GMM). Trypsin activity was expressed as total trypsin activity in International Unit (IU) $larva^{-1}$ and specific trypsin activity in IU μg^{-1} dry weight. For this purpose, the dry weight (DW) of *M. rosenbergii* larvae was used from Deru (1990). Samples of *P. elegans* (15 animals for each replicate and stage) larvae were washed in distilled water and oven dried on pre-weighed fine meshes at 60 °C for 24-36 h until constant weight was obtained. Measurement of the larval dry weight (in two replicates) was carried out as described in GMM, and the results are summarised in Table 1.

RESULTS

Survival and Growth

Palaemon elegans

Most of the larvae of *P. elegans* hatched at night during the full-moon. The larvae fed *Artemia* passed through 9 larval stages and developed into post-larvae within 12 days at a temperature of 25 °C and at 32.5 ppt S. The mean final survival and total length of the larvae were 88.5 % and 6.72 mm respectively. The PL+150 diet did not promote survival of the early larvae further than stage Z4. (Figures 1a, b). Although some larvae consumed the formulated diet and grew slightly better than starved controls (Figures 1a, b), complete mortality occurred on the 12th day of culture. Starved larvae perished within 6 days reaching only stage Z2.

When larvae previously fed *Artemia* were transferred at Z4-5 stages to artificial diet, they passed into stage postlarvae 1 (PL1) with a final mean survival of 49 % and 5.64 mm total length (Figures 2a, b). Mortality rate of larvae fed the artificial diet (4.73 % day^{-1}) was significantly ($P<0.001$) higher than that (0.74 % day^{-1}) of larvae fed live *Artemia*. Larvae fed *Artemia* displayed significantly ($P<0.001$) higher growth rate (0.304 mm day^{-1}) than those fed the artificial diets (0.103 mm day^{-1}). The development of the larvae fed formulated diets was retarded by 2-3 days. Larvae starved after stage Z4-5 survived only until stage Z7. Table 1 gives the dry weight of *P. elegans* and a comparison of its total length by different authors.

Macrobrachium rosenbergii

Since Brewster (1987) and Deru (1990) reported that *M. rosenbergii* cannot be reared on artificial diets during early stages, a Frippak CD3 microencapsulated diet (MED) was offered to the larvae only after Z5-6. These larvae developed from stage Z5-6 to PL1 within 16-17 days, but three days later than those in the *Artemia* control treatment. Figure 3a

shows that the mortality of MED-fed larvae was very high and larval growth was very slow between day 12 and day 20 possibly as a result of poor digestion during this period. Hence, their survival and growth were very similar to starved controls. After this adaptation period, their survival and growth curves followed those of larvae fed live *Artemia* nauplii (Figure 3b). Comparison of growth rates of larvae between day 20 and day 28 indicate that larvae fed artificial diet had an equal growth rate (0.352 mm day^{-1}) to those fed live *Artemia* (0.324 mm day^{-1}) during this period ($P > 0.05$). Finally, 28 % of the of larvae fed the artificial diets metamorphosed into PL1 with an average total length of 9.98 mm compared to 88.5 % mean survival of *Artemia*-fed larvae with an average length of 11.2 mm. The starved larvae moulted through only one further stage before dying on day 10. A high degree of cannibalism was observed among starved larvae and those fed capsules, particularly during the first week of the trial.

Feeding Behaviour and Gastroevacuation Time

The feeding behaviour of both species of larvae was observed during early and late stages. These caridean larvae generate a water current that brings the food particles and prey towards the mouth. Each time a live *Artemia* nauplius was captured by abdominal appendages, it was ingested completely before another one was caught. After ingestion, digestion, and excretion, which took approximately 20 min in early stage (Z2) *M. rosenbergii*, the larvae captured another *Artemia* nauplius. Larvae appeared to catch the prey by chance and capturing attempts were not always successful. When artificial particles were offered, *M. rosenbergii* larvae were observed to grasp and ingest the particles. However, unlike live *Artemia* nauplii, they did not consume the particles completely and some rejections occurred. The larvae (Z2) eagerly grasped particles when *Artemia* juice, prepared by crushing and filtering (20 μm) newly hatched nauplii, was added into the water with the artificial diet. *Palaemon elegans* early larvae appeared to be less selective in comparison to *Macrobrachium* larvae,

readily accepting artificial diet particles. Otherwise, the feeding behaviour of both species of caridean larvae was similar. When stage Z2 *Macrobrachium* larvae were stocked in three flasks (500 ml) and fed 10 *Artemia* ml^{-1}, 3.6 ± 0.69 ml^{-1} nauplii were left after 24 h. However, in control flasks stocked with only *Artemia*, 7.8 ± 1.22 ml^{-1} nauplii were found after 24 h. From these results, it may be concluded that the predation rate of *M. rosenbergii* larvae is circa 3.5 *Artemia* h^{-1} at stage Z2.

Continuous observation of some of the larvae for four h under the microscope also revealed that *M. rosenbergii* Z2 larva captured one *Artemia* naupli about every 20 min, equivalent to 2.7 nauplii h^{-1}. Following ingestion, charcoal labelled *Artemia* were visible in the gut and intestine of the larvae facilitating assessment of GET. It appears from Tables 2-3 that the GET of both species was quite similar. Early *M. rosenbergii* larvae (Z2-Z6) retained the food for about 10-20 min in their gut. However, the GET of late larvae was found to be 2-3 times longer (50 min at stage PL1) than for early stages. Similarly, *P. elegans* larvae also displayed a short GET (15-16 min) between Z2-Z6, and a longer GET during Z9-PL1 stages (30-50 min).

Trypsin Activity

Palaemon elegans

The larvae of *P. elegans* displayed low total (per organism) and specific (per μg^{-1} dry weight) trypsin activity between stage Z1 and Z4 (Table 2). The level of specific trypsin activity (about five fold) and total trypsin activity (7 fold) increased sharply at stage Z5 in comparison to that of stage Z1. After this peak, specific trypsin level declined steadily until Z9 stage. The relationship of trypsin activity to GET, and volume of hepatopancreas (HP) / organism dry weight (DW) are plotted together in Figure 4. This shows that during the first four zoeal stages, total trypsin activity, specific trypsin activity and HP / DW ratios of the larvae displayed the same trend.

A low HP / DW ratio corresponded to a low level of trypsin activity. At stage Z5, the 4-5 fold increase in trypsin activity coincided with a doubling in HP / DW ratio and the shortest GET. After the peak at stage Z5, specific trypsin activity declined sharply, although the HP / DW ratio continued to rise, but the larvae retained the food for longer periods in their gut.

Macrobrachium rosenbergii

As for *P. elegans* larvae, *M. rosenbergii* larvae also showed similar low total and specific trypsin activities during early stages (Z1-Z5) with a sharp increase (3-3.5 fold) at stage Z6 (Table 3). Specific trypsin activity for Z6 larvae was 6.35 x 10^{-5} IU μg^{-1} DW in comparison to 1.26 x 10^{-5} IU μg^{-1} DW for stage Z1. At Z6, this sharp increase in trypsin level occurred during a period when there was a large increase in HP / DW ratio (Figure 5) with the shortest GET. Although the pattern for total trypsin activity levels and HP / DW was similar for all larval stages, specific trypsin activity in the late larvae was very low (Figure 5) as a result of a large increase in body dry weight after stage Z5-6. During late larval stages, the filter apparatus is formed (Deru, 1990) and GET becomes much longer.

Figure 6 shows that trypsin activity during early stages of *M. rosenbergii* larvae (Z2 and Z4) starved, fed the Frippak diet and *Artemia* for 24 h was not significantly different ($P>0.05$). However, the late larvae (Z6 and Z9) fed artificial diet produced significantly ($P<0.05$) higher trypsin activities compared to those of starved and fed *Artemia*. In all cases, starvation for 24 h depressed larval trypsin activity.

Figures 1a, b. Survival (%) and total length (mm) of *P. elegans* larvae starved, and reared on two feeding regimes from Z1 to PL1 stage. Each symbol represents a mean ± s.d., n=2.

Figures 2a, b. Survival (%) and total length (mm) of *P. elegans* larvae starved and reared on two feeding regimes from Z4-5 to PL1 stage. Each symbol is a mean ± s.d., n=2.

Figure 1a

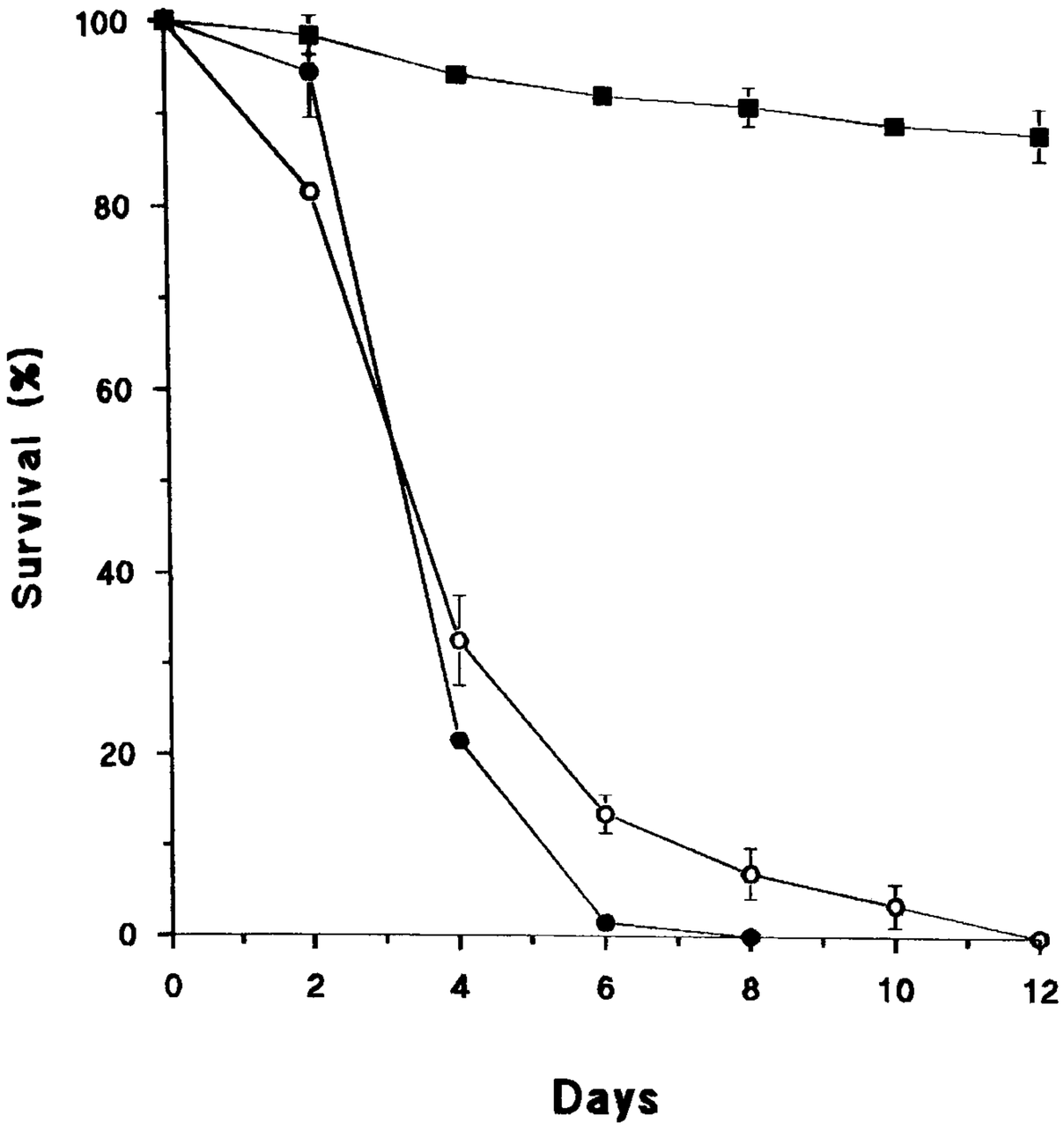


Figure 1b

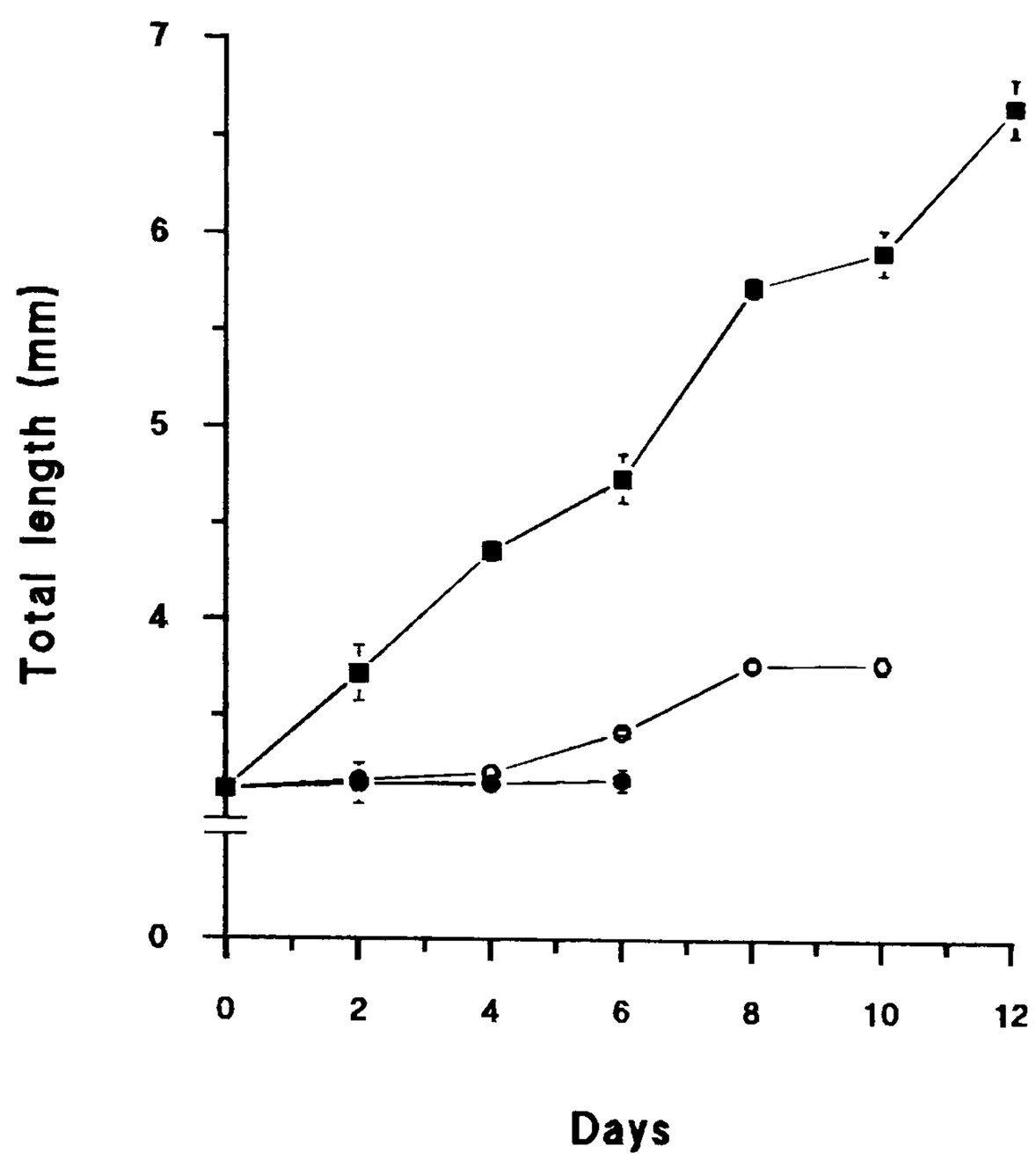


Figure 2a

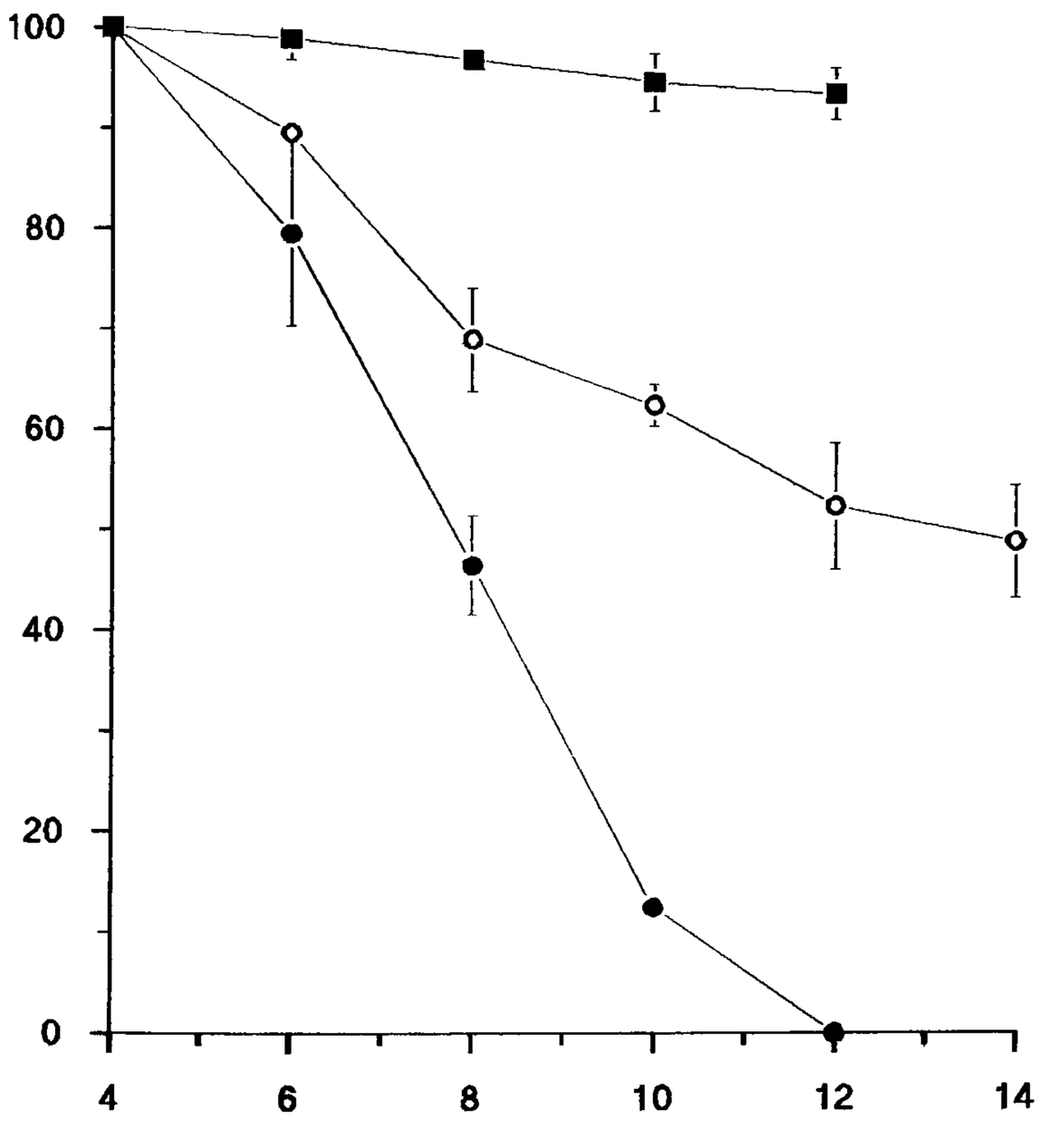
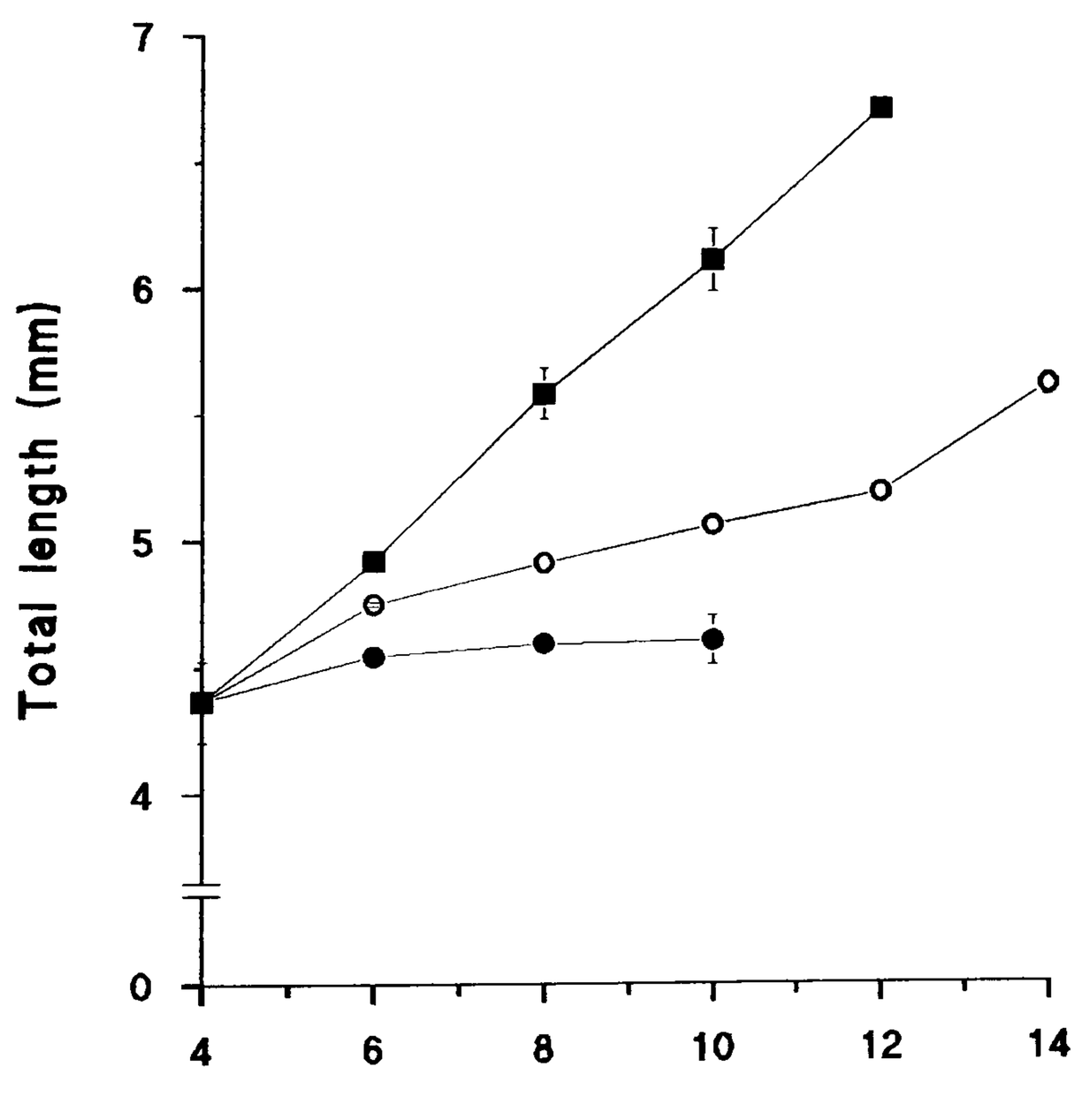


Figure 2b



■ Artemia ○ Artificial diet ● Starved

Figure 3a

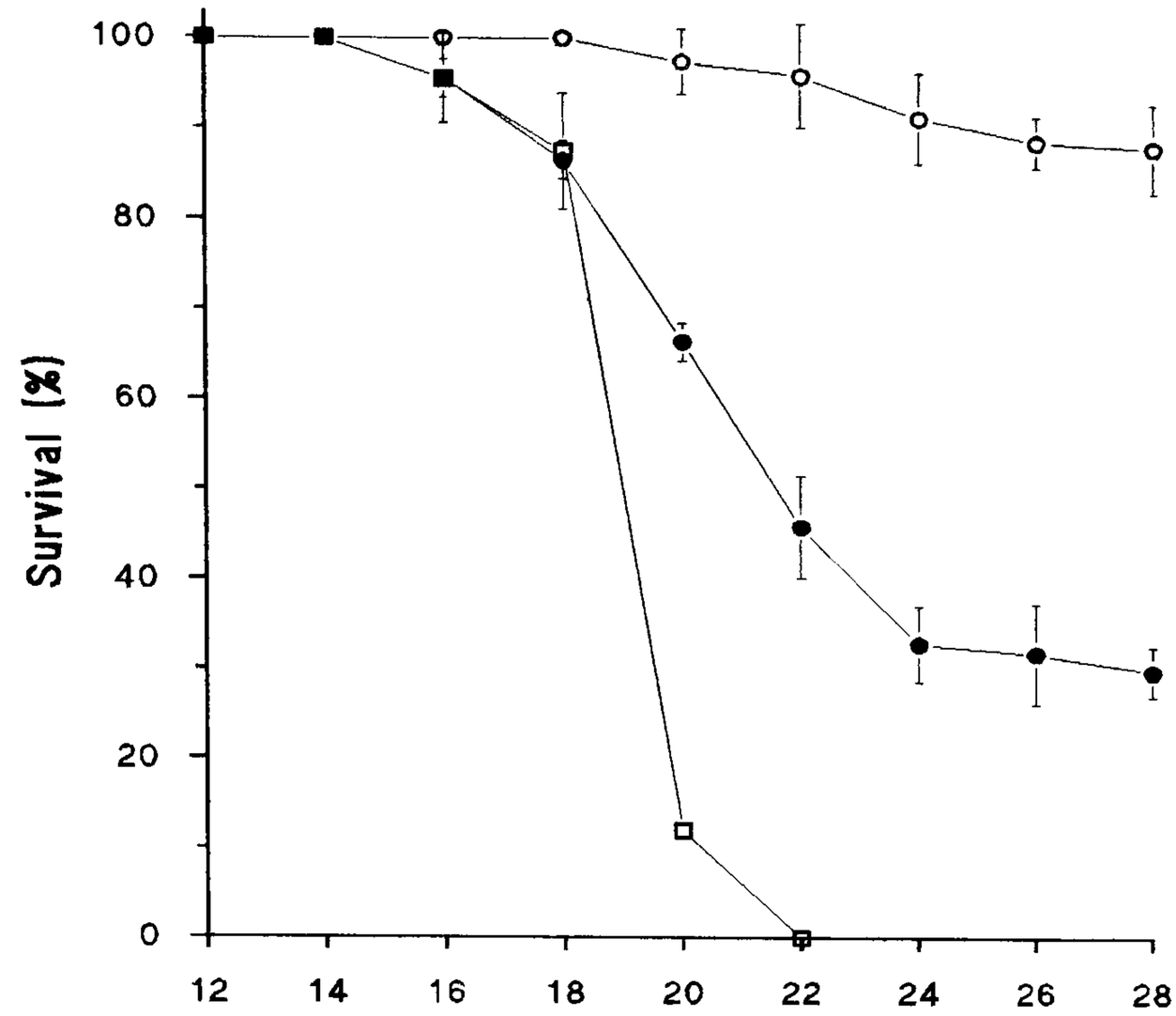
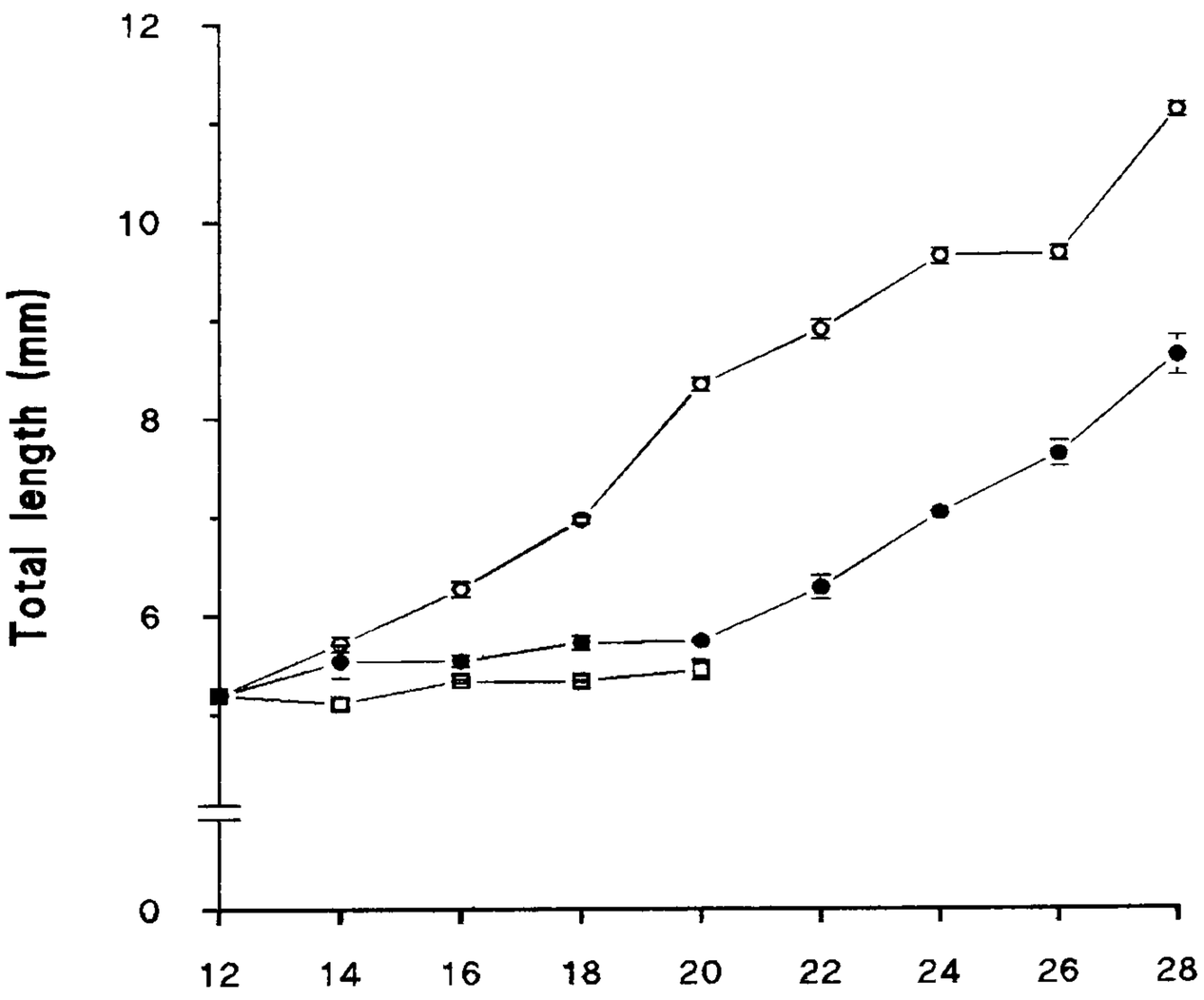
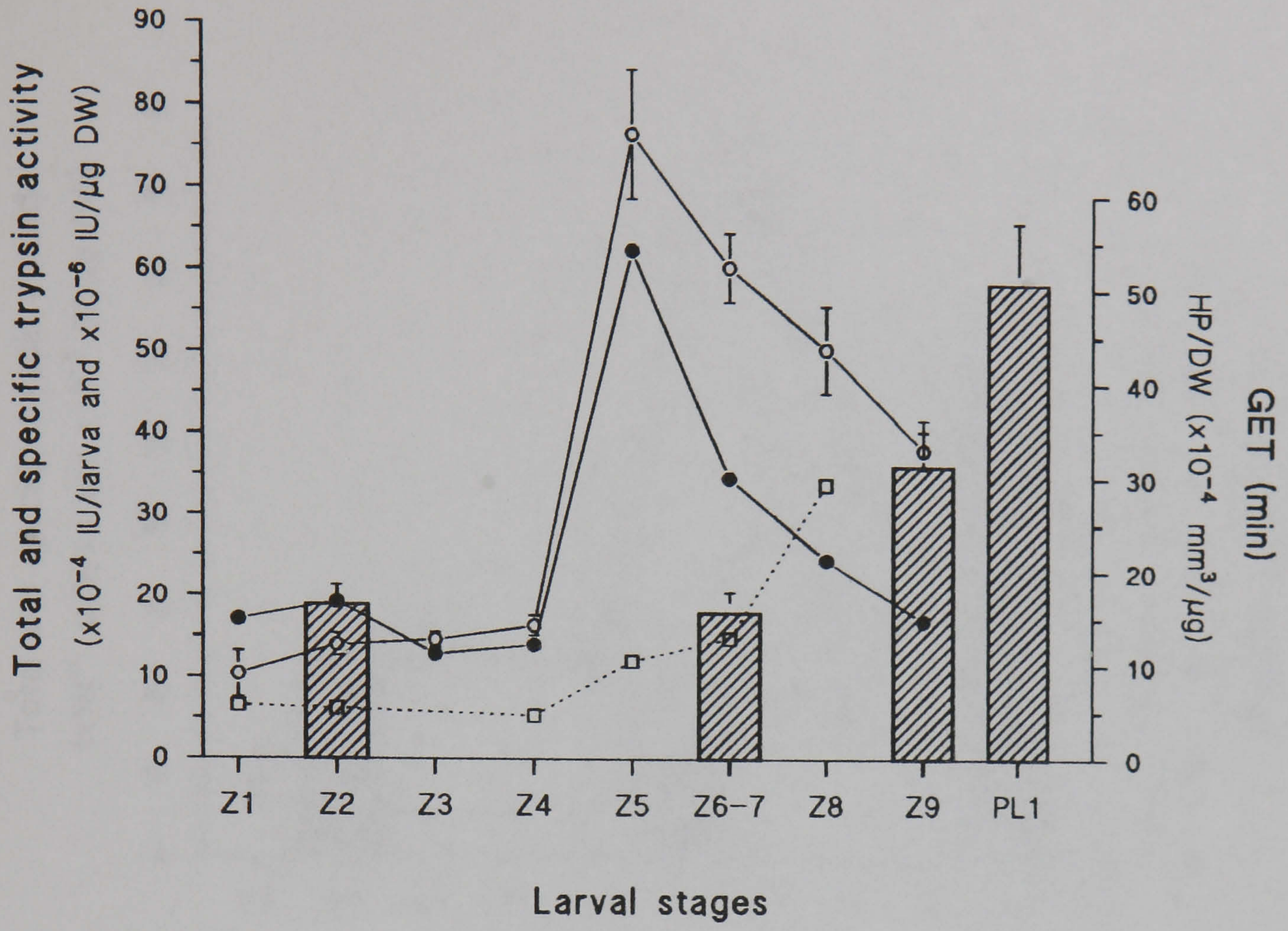


Figure 3b

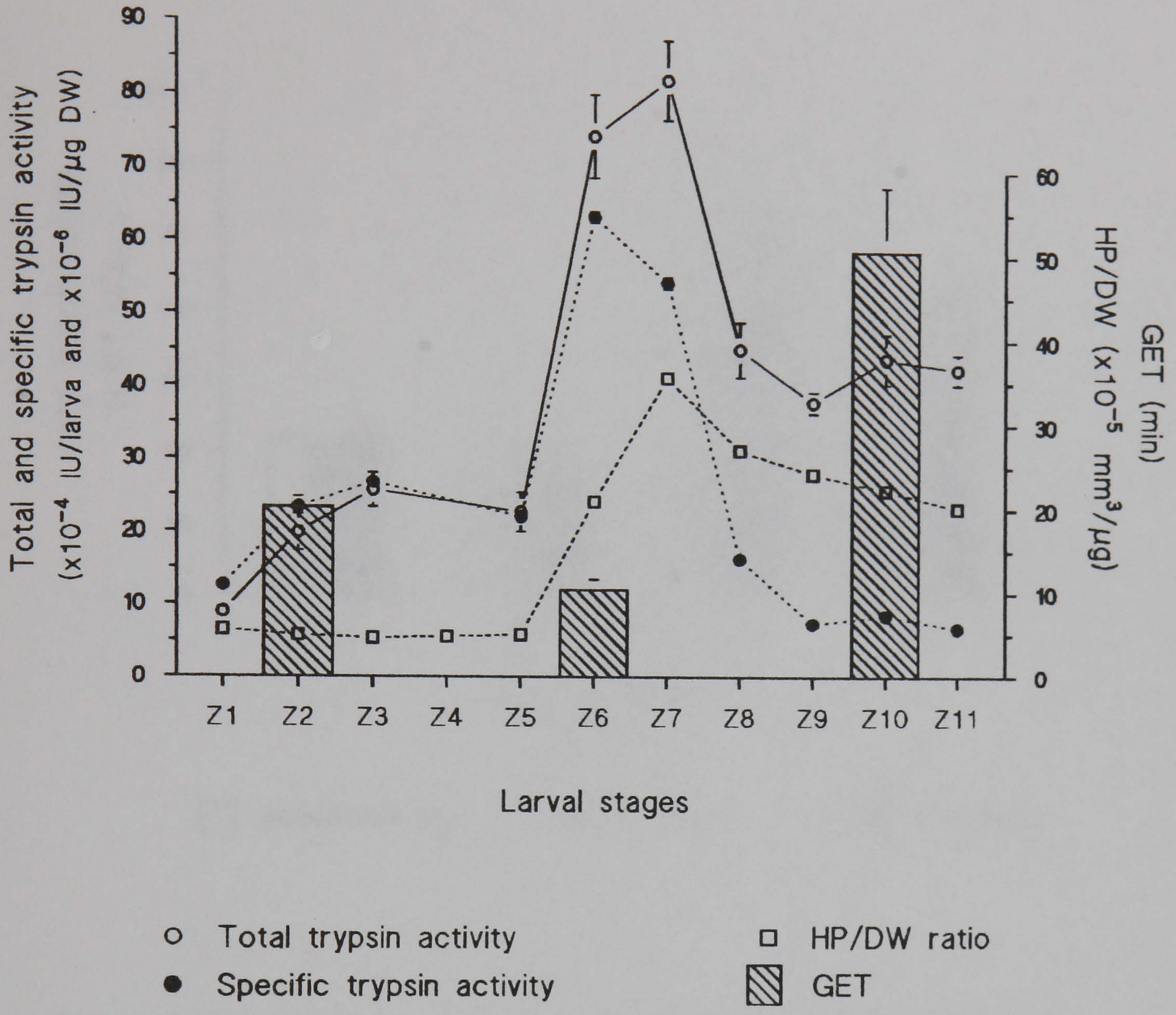


o Artemia ● Artificial diet □ Starved

Figures 3a, b. Survival (%) and total length (mm) of *M. rosenbergii* larvae starved fed *Artemia*, and artificial diet from Z4-5 to PL stage. Each symbol is a mean ± s.d., n=2.



Figures 4. Total (x10^{-4} IU/larva) and specific trypsin activity (x10^{-6} IU μg^{-1} dry weight = DW), volume of the hepatopancreas (HP) / DW ratio and gastroevacuation time (GET) for *P. elegans* larvae fed live *Artemia* nauplii throughout all larval stages. HP volume taken from Abubakr (1991). Each value for total and specific trypsin activity is a mean ± s.d. (n=2 for each treatment and n=3 for each assay).



Figures 5. Total ($x10^{-4}$ IU $larva^{-1}$) and specific trypsin activity ($x10^{-6}$ IU μg^{-1} dry weight = DW), volume of the hepatopancreas (HP) / DW ratio and gastroevacuation time (GET) for *M. rosenbergii* larvae fed live *Artemia* nauplii throughout all larval stages. HP volume and DW were taken from Deru (1990). Each value for total and specific trypsin activity is a mean ± s.d. (n=2 for each treatment and n=3 for each assay).

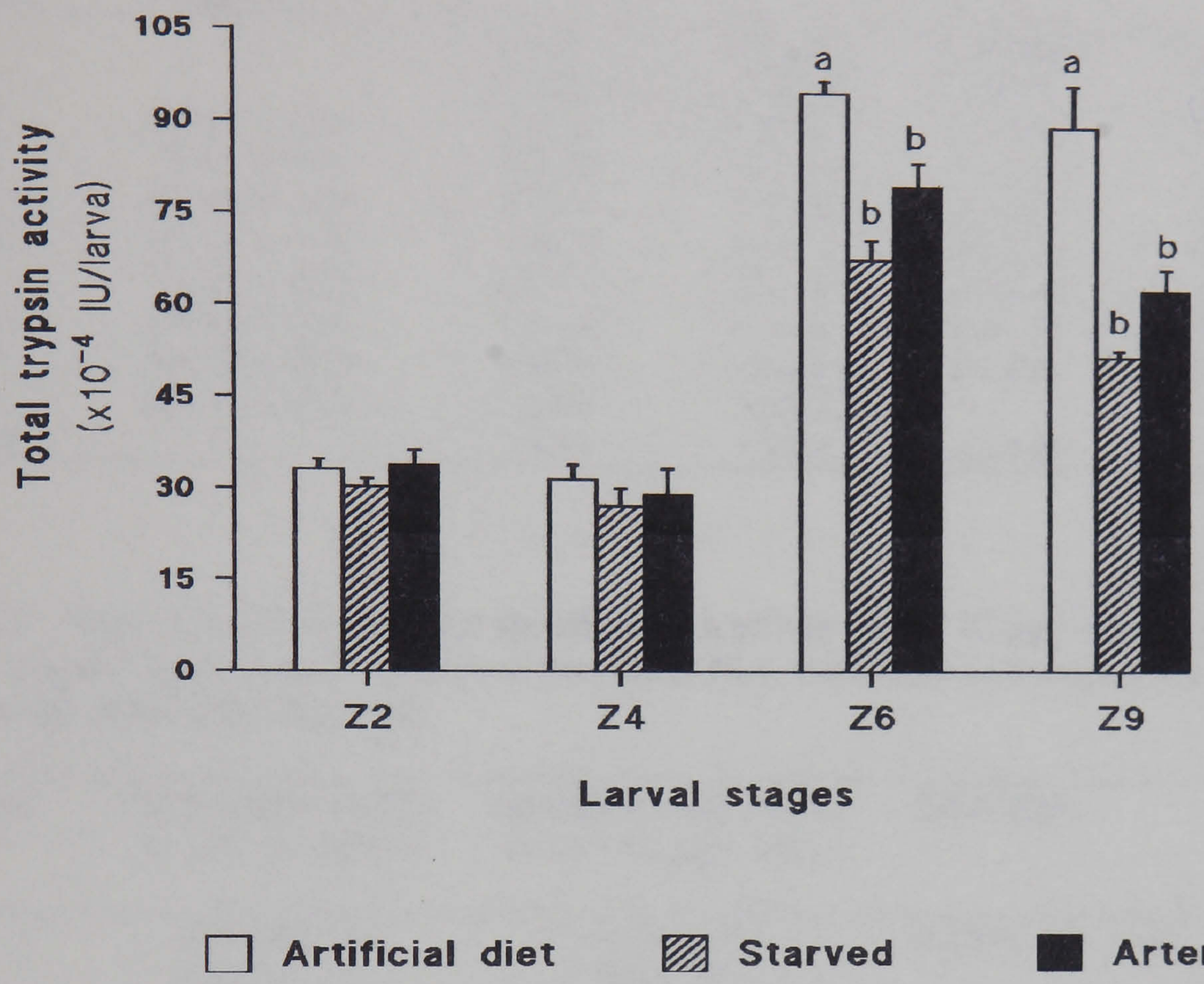


Figure 6. Total trypsin activity (x 10^{-4} IU $larva^{-1}$) of *M. rosenbergii* larvae starved or fed *Artemia*, and Frippak diet for a period of 24 h at early (Z2, Z3) and late larval stages (Z6, Z9). Each value is a mean ± s.d., n=2 for each treatment, n= at least 3 for each assay.

Table 1. Body dry weight (µg) and total length (mm) of *Palaemon elegans* larvae fed *Artemia* nauplii from Z1 to PL1. Each dry weight value represents a mean ± s.d., n=2 (15 animals for each stage and replicate).

Larval Stage	Dry weight (µg)	Total length (mm)			
		Present Experiment	Fincham (1977)	Tsurnamal (1963)	Ghamrawy (1976)
Z1	60.45 ± 0.64	3.0-3.2	2.8-3.2	2.2-2.80	2.6-2.8
Z2	72.65 ± 0.92	3.5-3.9	3.0-3.4	2.9-3.20	2.9-3.1
Z3	113.48 ± 0.73	4.1-4.5	3.3-3.8	3.6-3.75	3.5-3.7
Z4	116.95 ± 1.34	4.4-4.9	3.4-4.1	4.1-4.60	4.1-4.5
Z5	122.71 ± 0.12	4.8-5.3	4.2-4.8	4.3-5.40	4.7-5.3
Z6-7	174.63 ± 0.95	5.6-6.0	4.8-6.6	6.0-6.71	5.8-6.4
Z8	205.61 ± 2.23	6.0-6.5	6.0-6.6	6.5-6.75	6.3-6.9
Z9	223.40 ± 0.09	6.7-6.9	7.0-8.0	-	-
PL1	-	7.0-7.9	7.5-8.4	6.7-7.00	-

Table 2. Total (x 10^{-4} IU $larva^{-1}$) and specific trypsin activity (x 10^{-5} IU μg^{-1} dry weight = DW) of *P. elegans* larvae reared on *Artemia* from Z1 to PL1. Each value is a mean ± s.d. , n=2 for each stage and n=3 for each assay.

Stages	Total trypsin activity (x 10^{-4} IU $larva^{-1}$)	Specific trypsin activity (x 10^{-5} IU μg^{-1} DW)	GET (min)	
Z1	10.347 ± 2.886	1.712 ± 0.065	16.28 ± 2.138	(n = 7)
Z2	14.023 ± 1.369	1.930 ± 0.023		
Z3	14.640 ± 0.929	1.290 + 0.009		
Z4	16.426 ± 1.264	1.405 ± 0.052		
Z5	76.733 ± 7.867	6.253 ± 0.166	15.57 ± 2.149	(n = 8)
Z6-7	60.507 ± 4.192	3.465 ± 0.093		
Z8	50.534 ± 5.351	2.458 ± 0.062		
Z9	38.199 ± 2.305	1.710 ± 0.079	31.17 ± 4 .915	(n = 6)
			50.60 ± 6.473	(n = 5)

Table 3. Total (x 10^{-4} IU $larva^{-1}$) and specific trypsin activity (x 10^{-5} IU μg^{-1} dry weight = DW) of *M. rosenbergii* larvae fed live *Artemia* nauplii from Z1 to PL1. Each value represents a mean ± s.d., n=2 for each stage, and n=3 for each assay.

Stages	Total trypsin activity (x 10^{-4} IU larva)	Specific trypsin activity (x 10^{-5} IU μg^{-1})	GET (min)	
Z1	8.927 ± 0.578	1.257 ± 0.052		
Z2	19.878 ± 2.518	2.338 ± 0.362	20.00 ± 1.224	(n = 10)
Z3	25.729 ± 2.377	2.680 ± 0.429		
Z4	22.789 ± 2.685	2.213 ± 0.175		
Z5	74.425 ± 5.665	6.346 ± 0.738		
Z6	82.122 ± 5.451	5.438 ± 0.812	10.30 ± 1.259	(n = 8)
Z7	45.317 ± 3.788	1.630 ± 0.279		
Z8	38.037 ± 1.498	0.738 ± 0.019		
Z9	44.102 ± 3.434	0.856 ± 0.066		
Z10	42.689 ± 2.096	0.674 ± 0.062	50.50 ± 7.593	(n = 9)
Z11	52.613 ± 1.460	0.661 ± 0.018		

DISCUSSION

P. elegans larvae fed 15 *Artemia* nauplii ml^{-1} started to metamorphose into the PL1 stage within only 12 days at a temperature of 25 °C with over 88 % survival. Lower densities of live diet with lower temperatures are known to lengthen the duration of metamorphosis. For example, when Brewster (1987) fed *Palaemon* larvae on 10 *Artemia* nauplii ml^{-1} at 20 °C, the larvae metamorphosed into PL1 within 19-21 days. Yúfera and Rodríguez (1985) reported that *Palaemon serratus* larvae ingested more *Artemia* nauplii at 25 °C than at 20 °C. In the present study, high temperature did not seem to affect inversely the growth and survival of *P. elegans* larvae (see Table 1). *M. rosenbergii* larvae started to metamorphose into PL1 on day 24 when they were fed live *Artemia* (15 ml^{-1}) nauplii at 29 °C and 12 ppt (New, 1990). Moulting frequencies of both species of larvae were observed to be non-synchronised particularly after stage Z5-6.

P. elegans Z1 larvae fed artificial diet survived 12 days but never reached stage Z4. This is in agreement with Brewster (1987) who also failed to rear the early larvae on artificial diets. The latter author reported that the larvae were unable to survive on microencapsulated diets beyond day 9 and stage Z2-3. In the present study, although the larvae consumed the artificial diet and tended to retain it in their guts longer than *Artemia,* they were only able to survive 10 days, which was 2-3 days longer than starved larvae (Figure 1a). Similarly, *M. rosenbergii* larvae have not been reared successfully on artificial diets alone (Sick and Beaty, 1975; Murai and Andrews, 1978; El-Gamal, 1987) particularly during early larval stages (Deru, 1990). In current studies, an attempt to rear these larvae from Z5-6 on a MED resulted in 28 % survival until PL1 stage with 2-3 days retardation in larval development compared to *Artemia* fed control (Figure 3a). *P. elegans* late larvae (Z4-5), previously fed live diet, were also successfully reared until PL1 stage on a microgranulated diet (49 % survival) with a similar 2-3 days

retardation and slower growth (Figures 2a, b). Like penaeids, caridean larvae do not actively capture their prey, but rely on chance encounter and the abundance of food in the culture media (Jones et al., 1975; Moller, 1978). It is necessary to use large food particles when rearing these caridean larvae and this results in a high settlement rate of the food particles causing larval fouling and possibly bacterial growth (Jones et al., 1975). The absence of sufficient food particles may also reduce the ability of the larvae to catch sufficient food (New, 1990). Once a larva captures an *Artemia* nauplii, it is consumed completely before prey foraging is recommenced. In contrast, artificial particles were often only partially consumed and kept longer in the gut. When *Artemia* nauplii juice was added into the water, diet particle capture was more frequent suggesting that the artificial diet lacks attractants (Jones et al., 1975; Moller, 1978).

Trypsin is the dominant proteolytic enzyme in crustacean larval digestion (MacDonald et al., 1989; Lovett and Felder, 1990a) and is thought to be responsible for 40-60 % of total proteolysis in the hepatopancreas (HP) of *P. kerathurus* and *P. japonicus* (Galgani et al., 1984). Present results show that both *M. rosenbergii* and *P. elegans* larvae have low levels of trypsin activity between stages Z1 and Z4-5, but that these levels rise sharply (3-6 fold) at Z5-6 stages (Figures 4, 5). Similarly, Van Wormhoudt (1973) reported a sharp increase in protease activity at Z5 stage larvae of *Palaemon serratus*. It is known that diets influence digestive enzyme activity but since the same prey organism (*Artemia*) was given throughout all larval stages, the sharp increase in trypsin level at Z5-6 is related to development of the gut. Figures 4 and 5 demonstrate that this rapid increase in enzyme activity coincides with the rapid expansion of the hepatopancreas at Z5 in *P.* elegans, and at Z6 in *M. rosenbergii* larvae. It appears that the present results are in disagreement with Kamarudin et al., (1994) who reported high specific trypsin activities in *M. rosenbergii* larvae ranging from 13.2-30.84 x 10^{-5} IU μg^{-1} DW during early stages (Z1-Z5) to 7.7-9.13 x 10^{-5} IU μg^{-1} DW

during late larval stages (Z6-PL1). The present study demonstrates that average specific trypsin activity for early stages of *P. elegans* (1.58 x 10^{-5} IU μg^{-1} DW) and *M. rosenbergii* (2.09 x 10^{-5} IU μg^{-1} DW) is very low in comparison with specific trypsin activity of penaeid larvae such as *P. monodon* (Kumlu et al., 1992) and *P. japonicus* (Kamarudin, 1992; Le Vay et al., 1993), which ranges from 29-38 x 10^{-5} to 42.68 x 10^{-5} IU μg^{-1} DW, respectively, during protozoeal stages (PZ1-PZ3). This high digestive activity and short GET in penaeid larvae may be an adaptation to the lower energy natural food source of microalgae and to poorly digestible artificial diets (Jones et al., 1993).

Caridean larvae, particularly *P. elegans*, with guts full of artificial diets show poor growth and survival, suggesting that these larvae cannot digest or possibly assimilate enough artificial diet to supply growth requirements due to low levels of digestive enzymes during early stages. Lack of a gastric mill and filter apparatus until stage Z3 in *M. rosenbergii* (Deru, 1990) and Z4 in *P. elegans* (Abubakr, 1991) together with low trypsin activity dictates a requirement for an easily digestible food with a high energy content during early zoeal stages. Although trypsin levels of the larvae of both species were very low during early zoeal stages, *Artemia* fed larvae managed to survive without high mortality or depressed growth suggesting that this live diet fulfils all necessary physical and nutritional requirements and is easily digestible. Recently, Lovett and Felder (1990a, b) proposed that the decrease in endogenous enzyme production after stage M1 in *Penaeus setiferus* may be due to replacement by exogenous enzymes supplied from *Artemia* nauplii. Several authors also state that exogenous enzymes from live *Artemia* nauplii contribute to the autolysis of the prey and help digestion in fish larvae (Dabrowski, 1979; Munilla-Moran et al., 1990). However, contribution of exogenous enzymes from the prey (*Artemia*) to the digestion process of *M. rosenbergii* larvae and penaeid larvae has been measured and found to be insignificant (Jones et al., 1993; Kamarudin et

al., 1994). Although during late larval stages the HP / DW ratio continued to increase, trypsin production in both caridean prawns declined until PL1 when they were fed *Artemia*. The low levels of trypsin during late larval stages may be compensated for by retention of the food for longer periods in the more fully developed gut (Figures 4, 5). Mayzaud (1986) suggested that there may be a feed-back mechanism between the activity of digestive system and the energy demand of an organism, and that any change in energy requirement will induce an activation or repression of digestive enzymes. In the present study, although increase in volume of the HP coincides with a sharp increase in trypsin activity at stage Z5-6 (Figures 4, 5), dramatic increase in GET (3-5 times) during late larval stages may compensate for the need to increase digestive enzyme secretion during these stages when the larvae are feeding on easily digestible live *Artemia* (Le Vay et al., 1993).

In contrast to the situation in early stages, trypsin activity is enhanced in response to artificial diets during late larval stages. As Z6 and Z9 stages of *M. rosenbergii* fed artificial diet produced higher levels of trypsin activity than when fed live *Artemia* (Figure 6), and both feeds contain similar protein and energy levels (Amjad et al., 1992), it is likely that protein in artificial diets is less digestible. Similarly Jones et al., (1993), and Kamarudin et al., (1994) also reported higher trypsin activities for *P. monodon*, *P. japonicus* and *P. indicus* (see Chapter 5) when fed artificial diet rather than live diets during mysis stages. It appears that caridean late larvae exhibit a similar digestive strategy to penaeid mysis stages by elevating trypsin secretion when dietary protein is scarce or poorly digestible (artificial diets) and reducing secretion when protein is digestible and readily available (*Artemia*). Since proteolytic enzyme activity in early caridean larvae is low and stimulation of production levels does not appear to be possible, it is suggested that pre-digested and/or digestive enzymes should be incorporated into artificial diets for rearing these species.

CHAPTER 7

TRYPSIN ACTIVITY AS A TOOL TO DESCRIBE FEEDING STRATEGIES OF DECAPOD CRUSTACEAN LARVAE.

INTRODUCTION

Crustacean larvae employ different feeding strategies in the plankton to utilise food sources most efficiently until they metamorphose and become benthic. Newly hatched larvae first rely on their internal food sources for a few days, depending on the species, but once these sources have been depleted they require external feeds to meet their energy demands. With the exception of the early stages of penaeids (Wilkenfeld et al., 1984; Jones et al., 1993) most larvae of decapod crustaceans are predominantly considered as carnivorous (McConaugha, 1985).

Studies conducted in the laboratory have shown that the primary food varies according to the feeding mechanism of the species; carideans such as *Macrobrachium rosenbergii* (Ling, 1969a, b; Deru, 1990; Kamarudin et al., 1994) and *Palaemon elegans* (Rochanaburanon and Williamson, 1976; Brewster, 1987), lobsters such as *Homarus americanus* (Anger et al., 1985), *H. gammarus* (Kurmaly et al., 1990; Abubakr, 1991) and *Nephrops norvegicus* (Figueiredo and Vilela, 1972; Anger and Puschel, 1986) hatch as zoea and can directly feed upon zooplankton throughout larval stages. In one investigation, however, *Crangon nigricauda* has been shown to successfully develop into the metamorphosis solely on a live algal diet (Villamar and Brusca, 1987). Some decapod species are also known to change their feeding strategies during their larval development. For example, early planktotrophic stages of *C. maenas* feed initially on phytoplankton and shift to zooplankton during later stages (Williams, 1968; Rice and Williams, 1970). Similarly, studies with penaeids have shown that although these larvae are able to survive on zooplankton such as *Artemia* nauplii and nematodes throughout larval development (Wilkenfeld et al., 1984; Chapters 3, 4), they are best cultured on microalgae during zoeal and then on an animal prey (mainly *Artemia*) during mysis stages until the metamorphosis (Hudinaga, 1942; Emmerson, 1980; Yúfera et al., 1984).

Studies on larval digestive physiology help to understand the nutritional requirements and feeding ecology of an organism. It is known that digestive enzymes in larval decapod crustaceans are associated with development of the gut morphology (Jones and Kurmaly, 1987; Deru, 1990; Lovett and Felder, 1990a; Abubakr and Jones, 1992; Jones et al., 1993) and may be modified by diets (Kamarudin, 1992; Kumlu et al., 1992; Harms et al., 1994). Although there is a large body of literature related to gut morphology and digestive physiology of juvenile and adult decapod crustaceans (Yonge, 1924; Young, 1959; Dall, 1967; Van Weel, 1970; Barker and Gibson, 1977), research on larval gut morphology and digestive physiology is relatively recent. Lovett and Felder, (1989; 1990a, b) and Abubakr and Jones (1992) have extensively studied the larval gut morphology and digestive physiology of several penaeid species. The gut morphology of the caridean (*P. elegans* and *M. rosenbergii*), lobster (*H. gammarus*) and crab larvae (*C. maenas*) were investigated in detail by Deru (1990) and Abubakr (1991). The results revealed that decapod larvae have simple digestive systems, thus digestion is reliant upon their endogenous digestive enzymes with fast gastric emptying time for relatively less digestible feeds (e.g. algae or artificial diets, or for easily digestible prey e.g. zooplankton) and longer food retention time (Jones et al., 1993).

The studies with penaeid larvae (Kamarudin, 1992; Kumlu et al., 1992; Le Vay et al., 1993) have demonstrated that proteolytic digestive enzymes, in particular trypsin, which plays an important role in digestion of proteins in decapod crustaceans (Gibson and Barker, 1979; Galgani et al., 1984), has a high activity level and may be responsible with fast GET to enable the penaeids to survive solely on algae or artificial diets during larval development (Jones and Kurmaly, 1987). Digestive enzymes show considerable variation during ontogeny regardless of the type of diets. Investigations with caridean larvae *P. elegans* and *M. rosenbergii* in Chapter 6 suggest that, in contrast to penaeid larvae, these carnivorous larvae have limited

enzymatic capabilities during early larval development, and that increase in digestive enzymes at Z4-5 stages with longer food retention time may explain the ability of the late larvae to survive on relatively less digestible feeds (artificial diets) compared to zooplankton. Of the commercial species, only larvae with a high digestive enzyme activity have been successfully cultured on formulated diets to metamorphosis. To date, attempts to culture larvae that display low trypsin activity have always failed (Deru, 1990; Kurmaly et al., 1990).

Yonge (1937) stated that carnivorous crustaceans have more active proteases and weak carbohydrases compared to herbivorous ones. However, Degkwitz (1957: cited in Sather, 1969) found no relation between digestive enzymes and feeding mode of crustaceans. When Sather (1969) compared digestive enzyme activities in midgut of some crustaceans, he found that omnivorous and herbivorous animals show higher proteolytic enzymes compared to carnivorous ones. From the trypsin results obtained from the species studied in Chapter 5 (*P. indicus*), Chapter 6 (*P. elegans* and *M. rosenbergii*) and the literature (Kamarudin, 1992; Kumlu et al., 1992; Le Vay et al., 1993), it is proposed that determination of trypsin activity (IU μg^{-1} dry weight) may be a good indicator of the feeding strategy and the ability of crustacean larvae to survive on artificial diets. In addition to data available in the literature and previous chapters of this work, larval trypsin activity of two carnivorous species from the Family Nephropidae (*Homarus gammarus* and *Nephrops norvegicus*) and one brachyuran crab species (*Carcinus maenas*) were investigated at each stage of larval development through metamorphosis. Two calanoid copepod species (phytoplankton grazers) *Centropages typicus* and *Temora longicornis*, which do not belong to Order Decapoda, collected from the Menai Strait (UK) were also studied for this enzyme as they represent herbivorous or omnivorous grazers. Finally, specific trypsin activities of 11 different species larval crustaceans representing herbivorous, carnivorous and omnivorous are compared.

MATERIALS AND METHODS

Homarus gammarus

European lobster, *H. gammarus*, larvae were obtained from the Sea Zoo, Anglesey, UK. The larvae, which were feeding on frozen mysids in 100-l conical tank at 15-17 °C and 34 ppt S (as described in Beard and Wickins, 1992) were immediately (20 min) transferred to the School of Ocean Sciences, Menai Bridge, UK in 5-l sea water in a polythene bag in a styrofoam container. Larval stages were identified as described by Nichols and Lawton (1978) and sorted under a binocular microscope for the stages. 10 larvae for stage I, II, and III and 5 larvae at megalopa stage were taken into 1.5 ml microtest tubes for trypsin assays in duplicates. These samples were stored in a ultracold freezer (-74 °C) until the enzyme analysis. At least triplicate enzyme assays were performed for each larval stage and replicate. Trypsin assays were conducted with the method described in General Materials and Methods (GMM). Specific trypsin activity was obtained by dividing total trypsin activity by larval body dry weight obtained from Kurmaly (1989).

Carcinus maenas

Ovigerous *C. maenas* females were collected from the Menai Strait using a hand net. Ripe eggs were carefully removed from females using forceps and placed in shallow trays in sea water at room temperature of 18-19 °C for a few days. Newly hatched Z1 larvae were stocked in two 5-l round bottom glass flasks in filtered (0.2 µm) and UV treated sea water at room temperature and fed on 10 *Artemia* nauplii ml^{-1}. Everyday, 50% of the culture water was exchanged and larval stages were identified (Rice and Ingle, 1975). The numbers of larvae sampled for trypsin activity for each replicate were 100 for Z1, Z2, 70 for Z3, Z4 and 50 for megalopa stages. Samples were kept in a ultra-cold freezer (-74 °C) until the enzyme assays were con-

ducted. Two replicates for each stage and at least three replicates for each assays were performed for larval trypsin activity as described in GMM.

Nephrops norvegicus

Larvae of *N. norvegicus* were obtained from ovigerous females kept in a thermostatically controlled recirculation system at 14-15 °C and 34 ppt S at the School of Ocean Sciences, Menai Bridge, UK. Newly hatched larvae were fed on *Artemia* nauplii (5-10 ml^{-1}) in 2-l round bottom glass flasks until the samplings were performed. Two replicates of larval samples (10-15 larvae for each replicate) for stage I, II, and III were stocked in 1.5 ml microtest tubes for trypsin analysis and for body dry weight. Larval stages were identified according to Jorgensen (1925) and Figueiredo and Vilela (1972). The larval samples were kept in a ultra-cold freezer (-74 °C) until the assays were conducted for trypsin activity. Larval body dry weight and trypsin assays were determined by the method explained in GMM.

Centropages typicus and *Temora longicornis*

Two calanoid copepod species, *C. typicus* and *T. longicornis* were collected from the Menai Strait using a fine plankton net, which was towed slowly behind a boat for 10 min. The plankton samples were immediately brought to the laboratory (in 20-l bucket) where the zooplankton was concentrated on one side of the container and siphoned out into a 0.5-l glass beaker. The beaker was then kept on crushed ice to reduce the activity of the animals. A binocular microscope was used to select the most abundant calanoid copepod species placed on a counting tray. Among three species *C. typicus*, *T. longicornis* and *Paracalanus parvus*, the first two species were sampled for trypsin assays and body dry weight. 100 animals were sampled for each replicate (in duplicate) for the enzyme assays and body dry weight determination (GMM).

Larval trypsin results were compared using one-way ANOVA and Tukey's pairwise comparison test in Minitab.

RESULTS

Homarus gammarus

Figure 1 and Table 1 show total and specific trypsin activities of *H. gammarus* larva fed on frozen mysids during larval development. The larval total trypsin activity showed a gradual increase from stage I (67.19×10^{-3} IU $larva^{-1}$) to stage IV (92.84×10^{-3} IU $larva^{-1}$). Stage III and IV larvae had significantly higher trypsin activity than larvae at stage I and II ($P<0.05$). Specific trypsin activity of the larvae displayed a continuous decline from the first stage (3.07×10^{-5} IU μg^{-1}) to stage IV (1.12×10^{-5} IU μg^{-1}). Stage I larvae had the highest specific trypsin activity whereas stage IV larvae had the lowest ($P<0.05$). During stage II and III, specific trypsin activity of the larvae did not change significantly ($P>0.05$).

Table 1. Total, specific trypsin activity and larval body dry weight of *H. gammarus* larvae fed frozen mysids from stage I to stage IV.

Stages	Total trypsin activity (10^{-3} IU/larva)	Specific trypsin activity ($x10^{-5}$ IU/µg DW)	(*)Body dry weight (µg)
Stage I	67.185 ± 3.089	3.068 ± 0.741	2190
Stage II	75.586 ± 0.844	2.221 ± 0.368	3380
Stage III	87.398 ± 2.265	2.101 ± 0.373	4370
Stage IV	92.838 ± 4.793	1.129 ± 0.211	8270

(*) Values for larval dry weight were taken from Kurmaly (1989).

Nephrops norvegicus

Larval total and specific trypsin activities of *N. norvegicus* are presented in Figure 2 and Table 2. The data shows that trypsin activity per organism increased drastically from stage I to stage III. Increase in total trypsin activity between stage I and stage II was more than three fold, whereas the increase between stage II and stage III was 1.5 fold ($P<0.05$). Lowest specific trypsin activity was exhibited by stage I larvae. Specific trypsin activ-

ity of stage II larvae was almost 2.5 times more than larvae at stage II and 1.3 times more than larvae at stage III.

Table 2. Total, specific trypsin activities and body dry weight of *N. norvegicus* at stage I, II and III fed on *Artemia* nauplii.

Stages	Total trypsin activity ($x10^{-4}$ IU/larva)	Specific trypsin activity ($x10^{-5}$ IU/µg DW)	Body dry weight (µg)
Stage I	50.634 ± 6.097	0.850 ± 0.067	595.350±67.387
Stage II	165.927 ± 5.255	2.114 ± 0.059	785.000±15.633
Stage III	245.003 ± 16.613	1.638 ± 0.113	1495.553±62.930

Carcinus maenas

Figure 3 and Table 3 show total and specific trypsin activities of *C. maenas* during larval development through megalopa stage. Newly hatched non-feeding stage Z1 larvae displayed the lowest trypsin activity ($P<0.05$). When the larvae fed *Artemia* nauplii for a period of 48 h, trypsin activity per larvae significantly increased from 33.23 x 10^{-4} IU $larva^{-1}$ to 38.53 x 10^{-4} IU $larva^{-1}$. The larvae showed a very sharp increase in total trypsin activity between Z2 and Z4 stages. This increase was 61 % between Z2 and Z3 and 41 % between Z3 and Z4 stages.

Once the larvae metamorphosed into megalopa stage, however, trypsin activity dramatically dropped from 199.45 x 10^{-4} $larva^{-1}$ (Z4) to 36.38 x 10^{-4} IU $larva^{-1}$. Specific trypsin activity of the crab larvae did not show a great variation during larval development, except the megalopa stage. Z1 and Z4 stage larvae had showed significantly lower specific trypsin activity than that Z2 and Z4 stage larvae ($P<0.05$). The enzyme level did not significantly change between Z2 and Z4 stage. Megalopa larvae had 10-11 times less trypsin activity (µg^{-1} DW) in comparison to the previous larval stages.

Table 3. Total, specific trypsin activity and larval body dry weight of *C. maenas* during larval development.

Stages	Total trypsin activity ($x10^{-4}$ IU/larva)	Specific trypsin activity ($x10^{-5}$ IU/µg DW)	Body dry weight (*) (µg)
Z1	33.230 ± 0.039	17.235 ± 0.023	19.28
Z1(48 fed)	38.528 ± 3.209	-	-
Z2	87.954 ± 1.742	26.669 ± 0.052	32.98
Z3	141.550 ± 3.107	26.015 ± 0.075	54.41
Z4	199.450 ± 1.657	20.397 ± 0.034	97.78
Megalopa	36.281 ± 0.799	2.224 ± 0.047	163.13

(*) Dry weight values of the larvae were taken from Dawirs et al., (1986).

Centropages typicus and *Temora longicornis*

Total and specific trypsin activities of *C. typicus* and *T. longicornis* are presented in Table 4. Total trypsin activity (per organism) of *C. typicus* was relatively lower than that of *T. longicornis* (see Table 4). Similarly, the former displayed also lower trypsin activity per µg dry weight compared to the latter one. Dry weight of *T. longicornis* and *C. typicus* were 7.85 µg and 6.22 µg respectively (see Table 4).

Table 4. Total, specific trypsin activities and dry weight of *C. typicus* and *T. longicornis* collected from the Menai Strait, UK.

Species	Total trypsin activity ($x10^{-4}$ IU organism^{-1})	Specific trypsin activity ($x10^{-5}$ IU µg^{-1} DW)	Dry weight (µg)
Centropages typicus	25.058 ± 0.761	40.286 ± 1.235	6.22±0.282
Temora longicornis	41.730 ± 0.483	53.159 ± 1.015	7.85 ± 0.88

Figure 1. Total (x 10^{-3} IU $larva^{-1}$) and specific (x 10^{-5} IU μg^{-1}) trypsin activities in *H. gammarus* larvae fed on frozen mysids between stage I and stage IV. Each bar is a mean ± s.d. (n=3).

Figure 2. Total (x 10^{-4} IU $larva^{-1}$) and specific (x 10^{-5} IU μg^{-1}) trypsin activities in *N. norvegicus* larvae fed on *Artemia* nauplii between stage I and stage III. Each bar is a mean ± s.d. (n=3).

Figure 1

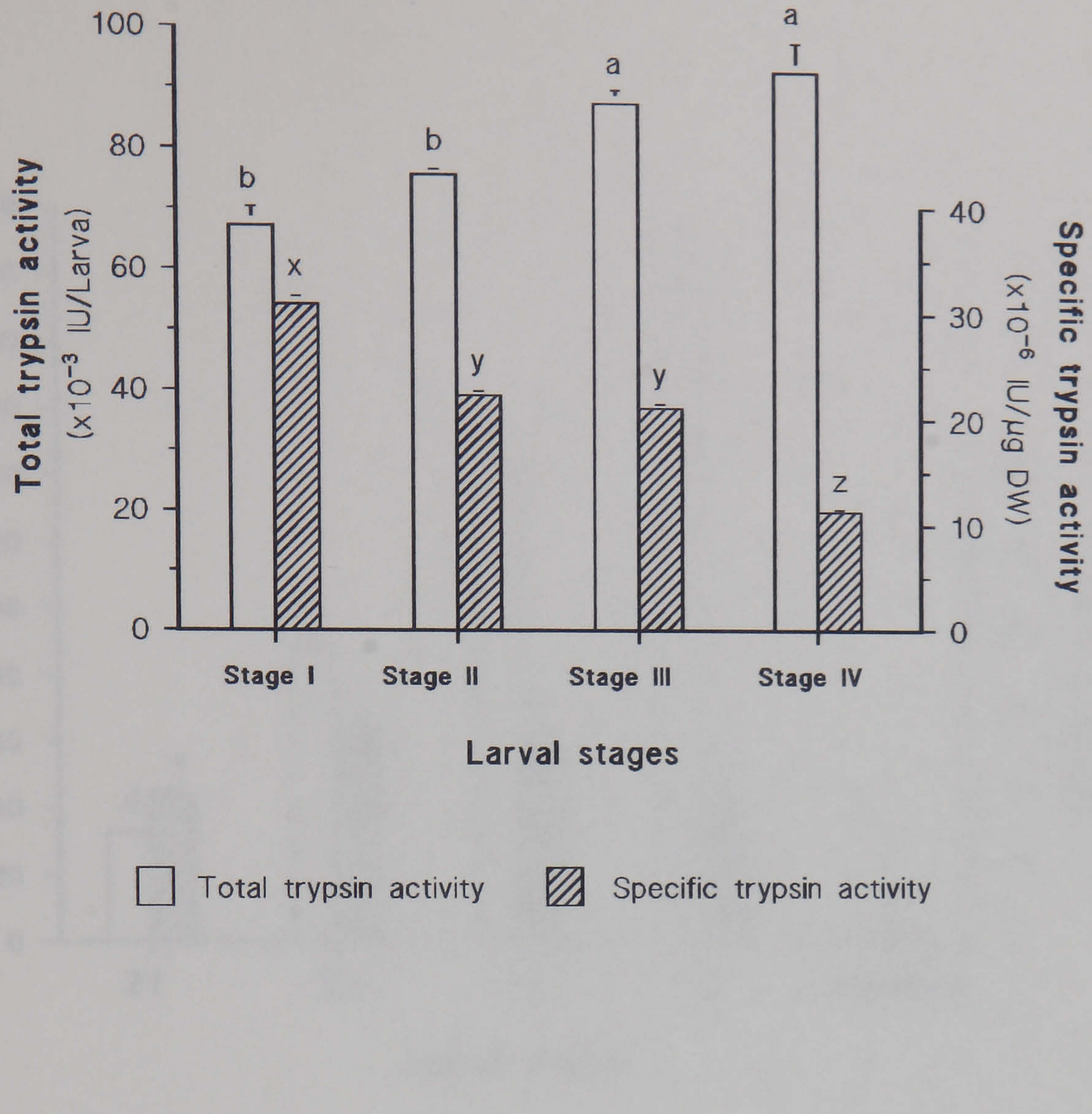
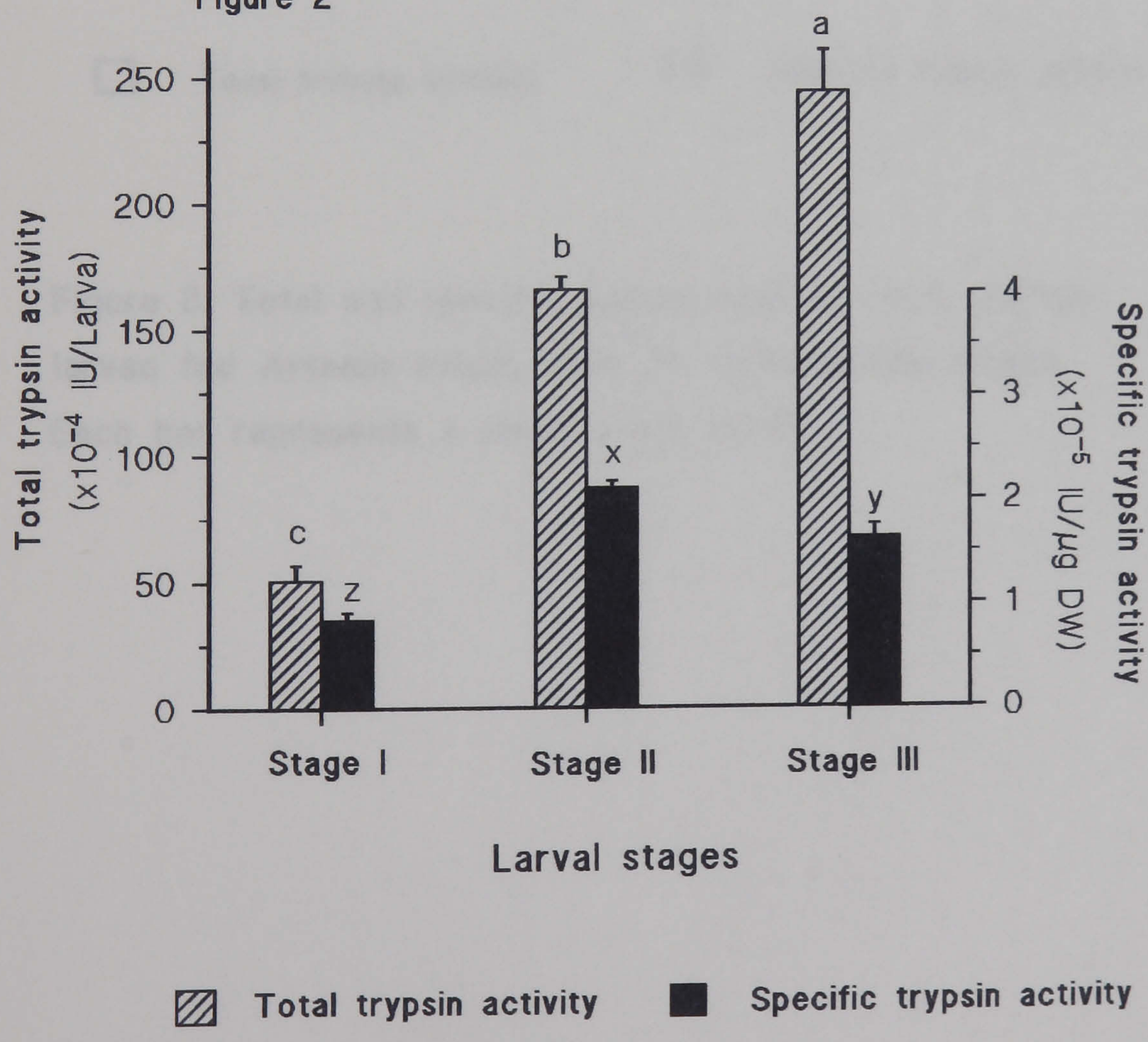


Figure 2



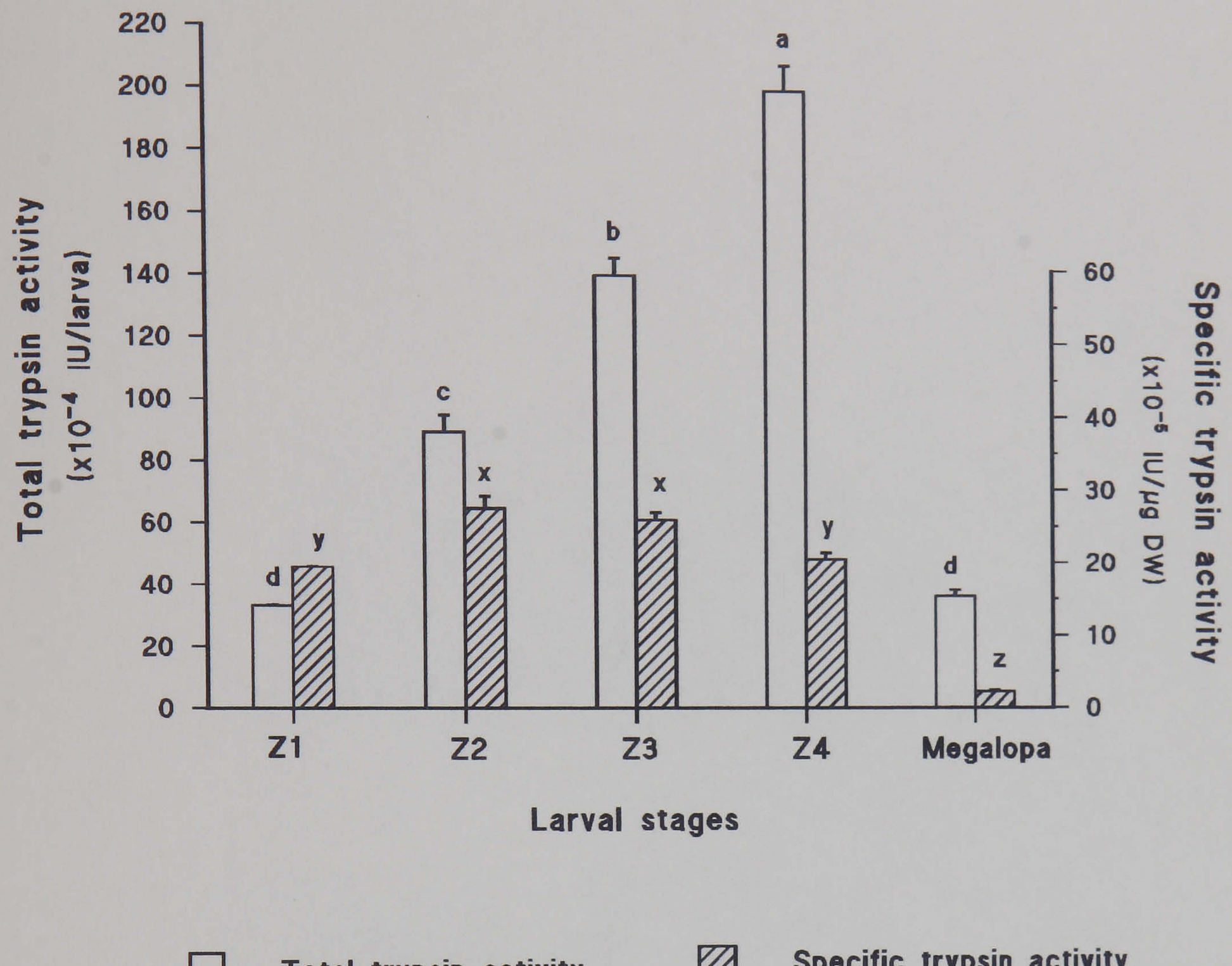
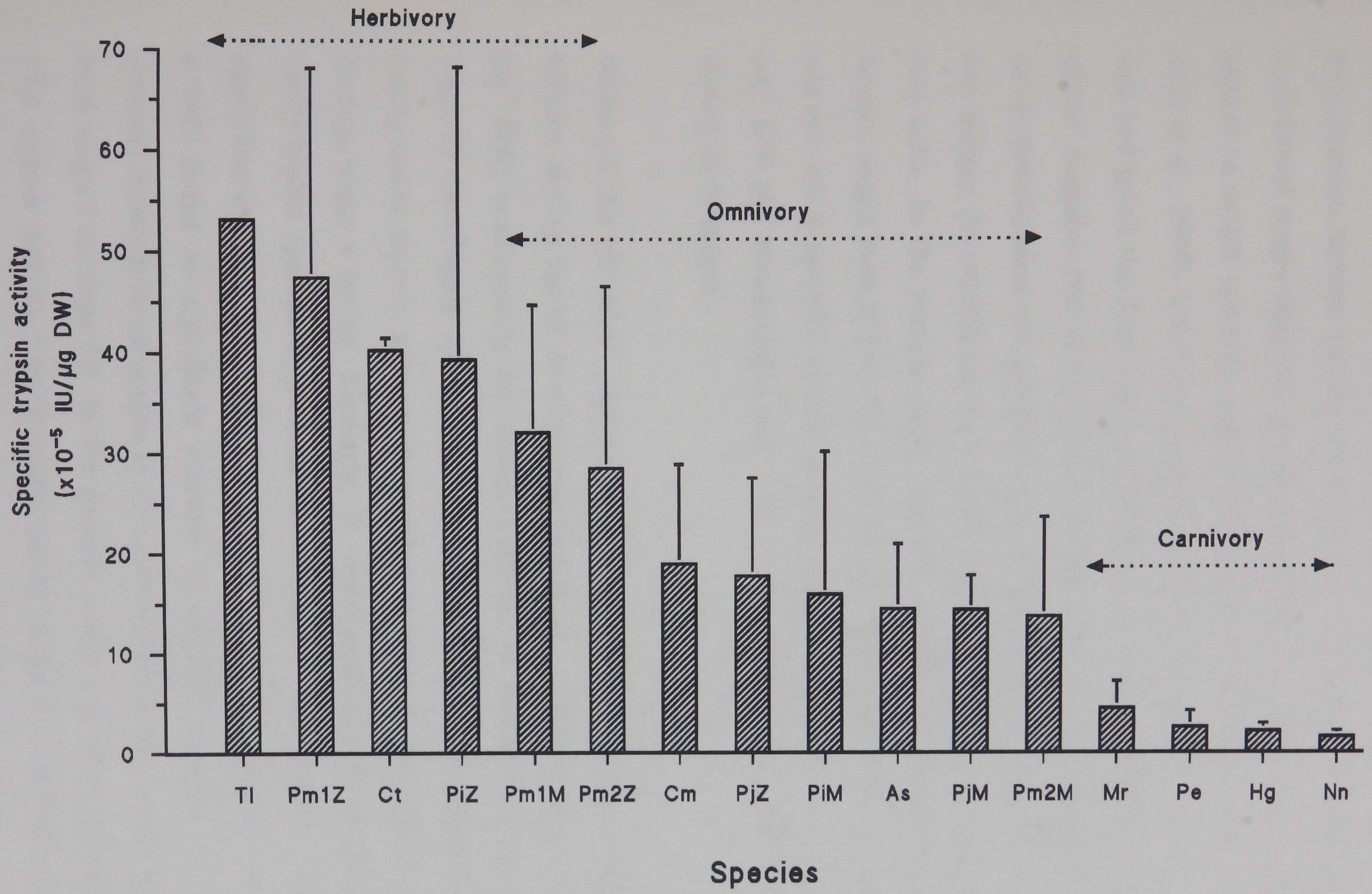


Figure 3. Total and specific trypsin activity of *C. maenas* larvae fed *Artemia* nauplii from Z1 to megalopa stages. Each bar represents a mean ± s.d. (n=2).

Figure 4. Comparison of specific trypsin activities (x 10^{-5} IU μg^{-1} DW) of different decapod crustacean larvae (except two calanoid copepod species) reared in the laboratory. Value of each bar is a mean of specific trypsin activity for larval stages described below for each species and ± s.d.

Abbreviation	Species	Stages	Authors
Tl	*T. longicornis*	-	(present study)
Pm1Z	*P. monodon*	(PZ1-M1)	(Kamarudin, 1992)
Ct	*C. typicus*	-	(present study)
PiZ	*P. indicus*	(PZ1-M1	(Chapter 5 in the present study)
Pm1M	*P. monodon*	(M2-PL1)	(Kamarudin, 1992)
Pm2Z	*P. monodon*	(PZ1-PZ3)	(Kumlu et al., 1992)
Cm	*C. maenas*	(Z1-Megalopa)	(present study)
PjZ	*P. japonicus*	(PZ1-M1)	(Kamarudin, 1992)
PiM	*P. indicus*	(M2-PL1)	(Chapter 5 in the present study)
As	*A. salina*	-	(Kamarudin, 1992)
PjM	*P. japonicus*	(M2-PL1)	(Kamarudin, 1992)
Pm2M	*P. monodon*	(M1-M3)	(Kumlu et al., 1992)
Mr	*M. rosenbergii*	(Z1-Z11)	(Chapter 6 in the present study)
Pe	*P. elegans*	(Z1-Z9)	(Chapter 6 in the present study)
Hg	*H. gammarus*	(Stage I-IV)	(the present study)
Nn	*N. norvegicus*	(Stage I-III)	(the present study)


Herbivory
Omnivory
Carnivory
Specific trypsin activity
(x10⁻⁵ IU/µg DW)
70
60
50
40
30
20
10
0
Tl
Pm1Z
Ct
PiZ
Pm1M
Pm2Z
Cm
PjZ
PiM
As
PjM
Pm2M
Mr
Pe
Hg
Nn
Species

DISCUSSION

Lobster larvae live in the plankton during their first three larval stages and then become benthic during the fourth or fifth stage. During their planktonic larval stages they rely on chance collision to capture prey and do not appear to orient towards suspended food (Hinton and Corey, 1979; Kurmaly et al., 1990). Until the gastric mill is developed at stage IV, the larvae tear and grind the food using their sharp mandibles (Abubakr, 1991). This author suggests that the main site for digestive enzyme production during larval development (*H. gammarus*) is the hepatopancreas (HP) as the anterior midgut diverticula are very small and do not appear to have any secretory cells. In the present study, specific trypsin activity of the nephropid larvae ranged from 0.85-3.07 x 10^{-5} IU μg^{-1} DW similar to the caridean larvae for which specific trypsin activity ranged from 1.26 to 2.21 x 10^{-5} IU μg^{-1} DW (*M. rosenbergii*) and 1.41-1.71 x 10^{-5} IU μg^{-1} DW (*P. elegans*) during early stages.

Although the HP of *H. gammarus* was shown to increase considerably in volume during larval development (Abubakr, 1991), trypsin activity (IU μg^{-1} DW) continuously decreased as the larvae developed from stage I to stage IV (see Figure 1). *H. gammarus* larvae at metamorphosis had significantly lower trypsin activity than either at stage II and III or even non-feeding stage I larvae. Similarly, *N. norvegicus* larvae exhibited significantly higher specific trypsin activity at stage II than stage I, but the level again decreased at stage III larvae (see Figure 2). Biesiot and Capuzzo (1990) found no significant increase in specific protease activity (mg^{-1} protein) between larval stages in *H. americanus* during larval development from stage I to stage IV. In the present study, a gradual decrease in specific trypsin activity was detected during larval development of both *H. gammarus* and *N. norvegicus*. This is attributed to a relatively faster increase in larval body dry weight than enzyme activity level during larval

zymes may also contribute to digestion in larval lobsters. Changes in the morphology of the mouth parts and the gut enables metamorphosed lobsters to deal successfully with more substantial food they encounter in the benthic habitat (Factor, 1981).

The crab, *C. maenas*, hatch as a non-feeding pre-zoeal larvae and develop through four zoeal stages and a megalopa stage until they reach the juvenile stage (Rice and Ingle, 1975). Earlier studies indicated that the larvae first feed on phytoplankton before they switch to zooplankton (Williams, 1968). Yet, as it was demonstrated in the present study, the newly hatched larvae can be successfully cultured on *Artemia* in the absence of algae throughout larval development. The larvae masticate the food by using their mouth parts during early stages until the gastric mill is formed at metamorphosis. Since the AMD and posterior midgut diverticula (PMD) constitute a very small proportion of the gut and do not show any secretory role as in penaeid larvae, digestive enzymes are produced by the HP, which continuously increases in its volume throughout larval stages (Abubakr, 1991). Trypsin results obtained in the present study show that digestive capability of *C. maenas* larvae is between that of herbivorous penaeid larvae and carnivorous lobster and caridean larvae. The larvae display significantly higher total trypsin activities with each successive moult to the next stage except for the megalopa ($P<0.05$).

In a recent study, Harms et al., (1994) also found a continuous increase in trypsin activity between Z1 and Z4 stages using the method of Samain et al., (1977). In the present study, the larvae fed on *Artemia* for 48 h had significantly higher trypsin activity compared to non-feeding larvae (Z1). Highest total trypsin activity was found in the larvae at Z4 stage. Specific trypsin activity of Z2 and Z4 larvae was higher than both Z1 and Z4 larvae. Either total or specific trypsin activity drastically dropped at the megalopa stage when the larvae switch from being planktotrophic to become benthic.

Since the same diet was given throughout larval development and the HP continues to increase in size (Abubakr, 1991), this drastic change in trypsin activity must be due to a change in feeding behaviour or feeding mode at the metamorphosis. Biesiot and Capuzzo (1990) also found a significantly lower protease activity at stage V in *H. americanus* and proposed that the reason for this might be related to changes in feeding behaviour or to physiological or biochemical factors or simply to the habitat change. Hirche and Anger (1987) state that the larvae prior to development into the megalopa stage do not necessarily need high digestive enzyme levels and may use the energy stored during previous stages as preparation for metamorphosis.

Even non-feeding Z1 larvae of *C. maenas* possess 9-10 times more trypsin activity (IU μg^{-1} DW) in comparison to carnivorous lobster larvae and this may enable them to utilise algae or even artificial diets during their early larval stages. In recent laboratory and field studies, several species of brachyuran and anomuran crab larvae have been shown to utilise algae and survive a few larval stages (Harms and Seeger, 1989). These authors report that although *Hyas coarctatus* were able to reach first juvenile crab stage on the algae, *Thalassiosira rotula*, *C. maenas* larvae moulted only to Z3-4 stages on the algal diets. However, large carnivorous larvae are unlikely to feed on small zooplankton or algae as effectively as smaller decapod larvae such as penaeids (Hudinaga, 1942; Emmerson, 1980). When penaeid larvae (see Chapter 5) shift from algae or artificial diets to relatively easily digestible feed (*Artemia*), they tend to reduce digestive enzyme secretion (Jones et al., 1993). Hence, although during Z1 and Z2 stages specific trypsin activity of *C. maenas* showed an increase, a gradual decrease between stage Z3 and Z4 may be explained by the easily digestible prey offered to them throughout larval stages. For the first time, Jones et al., (1975) reported that crab larvae accept and ingest microencapsulated diets. Kanazawa et al., (1983) reared the crab, *Portunus trituberculatus*,

larvae successfully from Z1 to juvenile stages. Levine and Sulkin (1983) also demonstrated that a brachyuran larva (*Eurypanopeus depressus*) is capable of capturing, ingesting and assimilating nutrients from calcium alginate microcapsules.

When the specific trypsin activity ($x10^{-5}$ IU μg^{-1} DW) of different larval crustaceans is plotted, a relation appears between the feeding strategy and the larval trypsin level (Figure 4). Trypsin values for each species were obtained by taking the mean of specific trypsin activity of all larval stages. Figure 4 indicates that there is a decline in trypsin activity from predominantly herbivorous larvae towards omnivorous and carnivorous larvae. Penaeid larvae are considered as herbivorous between PZ1 and M1 as they feed on microalgae, and omnivorous between M1 and PL stages as they feed on algae and zooplankton (Hudinaga, 1942; Langdon et al., 1985). A change in feeding strategy from being filter feeder to raptorial feeder is associated with morphological and physiological changes in the gut of the larvae during ontogeny. As the guts of herbivorous penaeid larvae lack masticatory filter apparatus, they maximise energy uptake by secreting high level of digestive enzymes with a fast gastroevacuation time (Jones and Kurmaly, 1987).

Unlike other decapods, these larvae possess anterior midgut diverticula (AMD) which are responsible for much of the digestive enzyme secretion during protozoeal stages although this organ gradually loses its function as the HP takes over at mysis stages (Abubakr and Jones, 1992). Figure 4 shows that penaeid larvae e.g. *P. monodon* (Kamarudin, 1992; Kumlu et al., 1992), *P. indicus* (Chapter 5), *P. japonicus* (Kamarudin, 1992) all display high trypsin activity during herbivorous stages, but enzyme levels decline once the larval feeding mode changes during mysis stages. Figure 4 demonstrates that specific trypsin activity of penaeid larvae range from 17.75 to 47.45 x 10^{-5} IU μg^{-1} DW during protozoeal stages.

Although penaeid larvae are known to be capable of consuming zooplankton such as *Artemia*, nematodes (Wilkenfeld et al., 1984; Chapters 3 and 4), rotifers (Emmerson and Andrews, 1981) as early as during first protozoeal stages, these feeds give a significantly lower survival and slower growth rate than microalgae. Several studies conducted with penaeids have indicated that the larvae can also be reared successfully on artificial diets without any live natural diets (Jones et al., 1987; Galgani and Aquacop, 1988; Ottogali, 1991). Figure 4 shows that the copepods, *T. longicornis*, *C. typicus*, which are known to be herbivorous or omnivorous (Marshal, 1973; Dagg, 1983), have high trypsin activities. Their main feeding mechanism may be filter feeding (Harris and Paffenhöfer, 1976). Specific trypsin activity of these copepod species were 53.16 (*T. longicornis*) and 40.29 x 10^{-5} IU μg^{-1} DW (*C. typicus*). The present results suggest that penaeids and copepods are adapted in their digestive enzymes in order to utilise a wide range of food sources in the plankton with a rapid gut turnover.

C. maenas larvae, which are considered as omnivorous at least during early stages, show an intermediate trypsin activity (18.97 x 10^{-5} IU μg^{-1} DW) between herbivorous and carnivorous larvae (see Figure 4). It appears that although the larvae were fed on *Artemia* nauplii solely, specific trypsin activity remained high during early stages and must be cued internally as suggested by Biesiot and Capuzzo (1990). Harms et al., (1994) suggest that phytoplankton is a major component of *C. maenas* larval diet and that their grazing activity may be similar to that in herbivorous larvae (Bautista and Harris, 1992). Although penaeid mysis larvae feed on zooplankton, they can be reared through metamorphosis on live microalgae but with slower growth and development. Omnivorous penaeid mysis and early PL's show relatively higher trypsin activity (between 14.37 and 32.11 x 10^{-5} IU μg^{-1} DW) than carnivorous but lower than herbivorous larvae. To ensure the optimum growth and survival these decapod larvae are best cultured on the mixed algae and zooplankton during mysis and early PL stages. The ability

to switch between phytoplankton and zooplankton during larval development gives a better chance of survival to these opportunistic larvae in the marine planktonic communities.

Figure 4 shows that carnivorous decapod crustaceans (e.g. *M. rosenbergii*, *P. elegans*, *H. gammarus* and *N. norvegicus*) have distinctly lower specific trypsin activities during larval development compared to herbivorous or omnivorous decapod larvae. This is in agreement with Yonge (1937), Sather (1969), and Brun and Wojtowicz (1976) who suggested that high protease and low carbohydrase activities are characteristic of carnivorous adult decapods whereas the inverse is true for herbivorous decapods. Omnivores are considered to be in between the two extremes. The specific trypsin activity of carnivorous larvae ranges from 1.53 to 4.46 x 10^{-5} IU μg^{-1} DW. Although some large diatom species may provide a additional source of nutrients for some carnivorous larvae (Harms and Seeger, 1989; Paul et al., 1989; Meyer-Harms and Harms, 1993), the larvae are large and require highly nutritional and easily digestible food to meet their energy demands.

Kurmaly et al., (1990) suggest that carnivorous lobster larvae (*H. gammarus*) cannot locate their prey at distance but are able to discriminate nutritious items from non-nutritious ones, and that this enables them to avoid continuously handling low-benefit food items. These authors also state that conditioning must play an important role in the feeding strategy of carnivorous larvae, not only to select nutritious items, but also those that are readily digestible. In contrast to penaeid larvae which secrete high amount of digestive enzymes and employ a very fast GET (5-7 times h: Kurmaly et al., 1989a), carnivorous decapod larvae have limited enzymatic capabilities but retain their food in their gut for longer periods to maximise assimilation efficiency and satisfy their energy needs (10-47 h for *H. gammarus* larvae: Kurmaly et al., 1990).

It appears that caridean larvae, however, have adopted a somewhat different strategy from that of the lobster larvae. Results of Chapter 6 revealed that since the early larvae have underdeveloped guts (Deru, 1990; Abubakr, 1991) and show low digestive enzyme activities and short GET, they may be entirely dependent on easily digestible feeds such as zooplankton. Yet, a sharp increase in trypsin activity, which coincides with a drastic increase in the volume of the HP, and longer gut retention time from Z4-5 onwards enable these larvae to cope with more substantial feeds such as microparticulated diets (Deru, 1990). Although the carnivorous larvae do not seem to have versatile and well-developed digestive systems, unlike herbivorous or omnivorous larvae, they can resist starvation for long periods when the prey is scarce. Anger and Dawirs (1981) suggest that crab larvae during larval development can accumulate enough energy to continue their development for a relatively long period of time independent of further food availability (Sasaki et al., 1986).

Recent studies have revealed that phytoplankton plays an important ecological role in nutrition of crab larvae in that it provides an additional food supply when zooplankton is scarce and may ensure a more balanced biochemical diversity of nutrients for the larvae (Harms and Seeger, 1989; Harms et al., 1991). The grazing activity of *C. maenas* has been compared to that of herbivorous copepods (Bautista and Harris, 1992) in that phytoplankton may constitute a major component of the natural diets of this species (Harms et al., 1994). However, the present results show that *C. maenas* larvae (24.15 ± 4.62 IU x 10^{-5} μg^{-1} DW) show lower trypsin activities than herbivorous copepods and are more similar to those omnivorous stages of penaeids (Kamarudin, 1992; Kumlu et al., 1992). Figure 4 shows the mean trypsin level (18.97 x 10^{-5} IU μg^{-1} DW) of *C. maenas* between Z1 and the megalopa stages. It appears that these larvae are capable of producing a trypsin level similar to other herbivorous-omnivorous decapod larvae such as *P. japonicus*, *P. indicus*, *P. monodon* and *A. salina* (see Figure 4)

even when *Artemia* nauplii are given exclusively. Hirche and Anger (1987) also state that amylase and trypsin activities of *Hyas araneus* are in the same range to those found in herbivorous copepods (Hirche, 1981).

Determination of trypsin appears to be a good indication to assess the feeding strategy and the ability of crustacean larvae to survive on artificial diets. However, trypsin activity of more decapod larval species representing different trophic levels should be investigated to see whether a majority of this taxa will fit the model proposed by the present study.

GENERAL CONCLUSIONS

In agreement with Aquacop (1983; 1984), present study shows that *P. indicus* can easily mature and spawn in captivity in recirculation systems. This provides an opportunity to close the life cycle of the species to obtain disease free nauplii throughout the year. It has been possible to culture *P. indicus* for 2-3 generations and to supply larvae regularly from the brood-stock originating from both India and the Red Sea (recently imported) in the School of Ocean Sciences, Menai Bridge, UK. Hence, it is suggested that using this species may enable the hatcheries to become independent of wild-caught gravid females and help preserve the wild stocks. Closing the life cycle of a species provides regular material for nutritional studies and also helps to improve the performance of cultured stocks (Csawas, 1994).

Chapter 1 demonstrates that *P. indicus* larvae show better growth, survival and development on the mixed algal feed, a flagellate species, *T. chuii* plus a diatom species, *S. costatum*, than either of the single algal species. This is expected to be due to a better nutritional composition of the mixed algal feeding regime than the single ones (Kurmaly et al., 1989a; Amjad, 1990). The optimal cell density appears to fall in between 60 and 70 cells μl^{-1} (25 cells μl^{-1} *T. chuii* and 35-45 cells μl^{-1} *S. costatum*). Cell densities lower than 70 cells μl^{-1} result in lower survival, slower growth and retardation in larval development, whereas higher cell densities pollute larval culture conditions. The diatom, *S. costatum*, as a single live feed supports high survival, growth and larval development in *P. indicus* at cell densities between 70 and 80 cells μl^{-1}. However, aggregation of the cells at these high cell concentrations may hamper active swimming and feeding of the larvae. Therefore, use of *S. costatum* as a single diet in the culture of *P. indicus* is not recommended as it may risk the entire hatchery operations (Liao et al., 1983; Jones et al., 1987). In contrast to results achieved with *P. monodon* (Kurmaly et al., 1989a; Amjad, 1990; Kamarudin, 1992)

with similar experimental conditions to those used in the present experiments, *R. reticulata* neither as a sole diet nor with other algal species is suitable for the culture of *P. indicus* larvae. The results show that this alga is ingested and digested by the shrimp larvae. As discussed in Chapter 1, the reason for the inability of the larvae to survive on this alga may lie in its nutritional composition. The results of Chapter 1 also demonstrate that *P. indicus* show preference to low salinities (20-25 ppt) during larval development. Optimal larval salinity requirement for *P. indicus* originating from India appears to fall in between 20 and 25 ppt S (PZ1 to PL1 stages). In conclusion, Chapter 1 demonstrates that at 25 ppt salinity, *P. indicus* larvae can be reared on *T. chuii/S. costatum* (60-70 cells μl^{-1}) plus five *Artemia* ml^{-1} with over 90 % survival and fast development (only 6-7 days) from PZ1 to PL1 stage.

Chapter 2 shows that *P. indicus* early postlarvae (PL7) reared at 34 ppt S require an adaptation period of approximately 48 h before stocking into waters of 10 or 5 ppt S. These PL's can successfully tolerate a 10 ppt S change without acclimation. Similar to larval stages, optimal culture salinity for Indian postlarvae, between PL7 and PL60, seems to be between 20-25 ppt S. High salinities (40 and 50 ppt) reduced survival, growth and give inferior increase in biomass per mm TL in comparison to lower salinities. The present results also demonstrate that although the early PL's grow and survive well at low salinities (5-10 ppt), these saline conditions are lethal to the postlarvae after PL40-45 stage. Comparison of results of the present study to those of Bukhari et al., (1994) suggests that despite similar optimal larval salinity requirements, postlarvae of *P. indicus* originating from India show different salinity preference to those from the Red Sea (Bukhari et al., 1994). These authors report that the Red Sea *P. indicus* postlarvae show better growth, survival and biomass in high salinity waters (43-50 ppt S).

Chapter 3 and 4 show that while the free-living nematode, *P. redivivus*, is not suitable as a food source to rear caridean larvae, they can be successfully used for the total replacement of live algae and *Artemia* in culture of *P. indicus*. Both *M. rosenbergii* and *P. elegans* employ a different feeding strategy compared to *P. indicus*. These caridean larvae are incapable of capturing and ingesting enough nematodes to satisfy their energy requirements due, possibly, to the shape and size of the prey. *P. indicus* larvae, even at PZ1 stage, are capable of ingesting and digesting the nematodes. The results indicate that a nematode concentration of 30 individuals ml^{-1} between PZ1 and PZ3, 45 nematodes ml^{-1} between M1-M2, and 60-70 nematodes ml^{-1} between M3 and PL1 are adequate to promote satisfactory larval survival, growth and development in *P. indicus*. Higher nematode densities than those above do not provide any advantage and may pollute culture conditions. In order to keep the nematodes (particularly large ones) in suspension for longer period of time, it is suggested that the nematode rations should be distributed at least twice a day. Also, higher aeration rate will help to reduce nematode settling rate. Introduction of small or large size *P. redivivus* do not markedly affect performance of *P. indicus* larvae during protozoeal or later stages.

As shown with other penaeid species e.g. *P. vannamei*, *P. setiferus* and *P. aztecus* (Wilkenfeld et al., 1984; Biedenbach et al., 1989), the nematode *P. redivivus* can be successfully used as an *Artemia* replacement during mysis and early PL stages of *P. indicus*. When *P. indicus* larvae, at the first feeding stage, are fed directly on the nematode, they show better survival, but generally slower growth and development compared to those fed on algae/*Artemia*. This may be due to relatively indigestible cuticle and possibly inferior HUFA content of the nematodes. Results of Chapter 4 demonstrate that provision of live algae (only 15 cells μl^{-1} for 24 or 48 h) as a co-feed with the nematodes stimulates significantly higher larval trypsin activity (at PZ1 stage) in comparison to the nematodes fed as a sole diet. Results indicate that larval growth and development on the single nematode feeding

regimes can equal those fed control live algae by the addition of 15 cells μl^{-1} during zoeal stages. Therefore, it is suggested that if it is available, providing a small amount of algae with the nematodes will ensure equal, if not better, larval survival, growth and development to those fed algae/*Artemia* for the culture of *P. indicus*.

As the main purpose of larval nutritional studies in this thesis is to totally eliminate the use of microalgae in penaeid culture, other alternative methods are investigated in Chapter 4. In agreement with Kahan et al., (1980) and Rouse et al., (1992), results of Chapter 4 show that biochemical composition of the nematodes can be manipulated by loading the alimentary canal with growth factors such as lipids, HUFA, and pigments. Although the nematode, *P. redivivus*, contains high levels of protein, it may lack important HUFA (e.g. 20: 5ω-3 and 22: 6ω-3) that are required in the diet for marine shrimps (Biedenbach et al., 1989). Present study shows that lipid-enrichment significantly increases the levels of long chain fatty acids, particularly 20: 5ω-3 and 22:6ω-3 of the HUFA, contents of the nematodes in comparison to non-enriched nematodes. The lipid-enriched nematodes result in better larval survival in *P. indicus* larvae compared to those non-lipid enriched nematodes. However, the lipid-enrichment has a less pronounced effect on larval growth and development.

If nematodes are to be used for totally replacing the conventional live diets in commercial penaeid hatcheries, the pale colour of the postlarvae (PL) normally obtained with nematode feeds must be improved. This problem may be overcome by feeding penaeid larvae on nematodes plus a low amount of live algae or on nematodes which are filled with a pigment source (astaxanthin). In the present study, both methods are demonstrated to be effective on pigmentation of *P. indicus* larvae. Despite several experiments conducted to determine any positive effect of the pigment (astaxanthin) on the growth of *P. indicus* larvae in the current study, the results are inconclusive. Yet,

pigmented nematodes consistently give significantly better larval survival over non-pigmented ones. It is proposed that use of either algae or pigment with the nematodes will depend on how much extra cost and labour they will add to overall expenses in PL production. As discussed in Wilkenfeld et al., (1984) and Chapter 3, live nematodes offer several practical advantages over algae and *Artemia*. Recently, it has been suggested that they can be cultured easily on various types of cheap liquid medium which allows them to be produced in mass quantities in large vessels suitable for commercial purposes (Fisher, pers. comm.). In the view of the present laboratory studies, the use of live nematodes in commercial hatcheries will obviously depend on their cost, availability and that how practical they may be to the farmers. Prior to any economic analysis, it is recommended that the nematodes have to be tested in feeding trials in commercial hatchery levels.

The ultimate aim of the larval nutritionists is to replace live algae and *Artemia* in culture of penaeids with an artificial diet which will be stable in culture water for long periods, cost-effective, storable for long period of time, acceptable and digestible to an organism with an appropriate nutritional content (Jones et al., 1993). Results of Chapter 5 reveal that microencapsulated diets are still far from being ideal to promote comparable larval survival and growth in penaeid culture. Several studies have shown that the diets are at correct particle size for ingestion, available in suspension at all times, and of a similar nutritional content to natural diets (Kurmaly et al., 1989a; Amjad et al., 1992; Jones et al., 1993; Le Vay et al., 1993). However, in agreement with Jones et al., (1993) and Le Vay et al., (1993), the present results suggest that the current microencapsulated diets (MED) may relatively be indigestible, lack gut enzyme stimulants and some nutritional components which may leach into the water. Current microcapsules retard larval development of *P. indicus* by 1-2 days until metamorphosis resulting in lower survival and growth rate compared to algae/ *Artemia* feeding regimes. Similar to the results obtained in Chapter 4, when 15 cells μl^{-1} frozen

algae is fed to *P. indicus* larvae with these microcapsules, a significant increase in larval trypsin activity occurs throughout all protozoeal stages compared to when microcapsules are fed as a sole diet. Increase in trypsin activity is accompanied by improvements in larval survival and growth and development. Poor digestibility of the diets causes high larval mortalities at very early larval stages (PZ1) when the internal energy sources have just been depleted and digestive capability of the larvae is still limited. It is thought that provision of algae at this stage may be critical in stimulating digestive enzymes which enable penaeid larvae to adapt to less digestible protein sources. At mysis stages, the effect of microalgae as a gut enzyme stimulant is less pronounced as the larvae already have high digestive enzymatic capabilities when volume of the AMD is maximum (Abubakr, 1991; Abubakr and Jones, 1992).

Once the larvae shift to feed on *Artemia* during mysis stages, larval trypsin production decreases as the prey provides an easily digestible and assimilatable protein (Lan and Pan, 1993). In contrast, larvae which are fed on MED as a sole diet at mysis stages maintain higher trypsin activity until the metamorphosis. This suggests that penaeid mysis larvae are capable of physiologically adapting their digestive enzymes according to the availability of dietary protein in the feed they are reared on. Similar to penaeids, results of Chapter 6 also demonstrate that the inability of caridean larvae, *P. elegans* and *M. rosenbergii*, to survive on formulated diets is due to poor digestibility of these diets. Caridean larvae, unlike penaeids, show limited enzymatic capabilities, particularly during early larval stages, when they cannot be cultured on artificial diets. However, present studies show that the larvae of both these caridean species are capable of utilising artificial diets from Z5-6 stages onwards when their trypsin activity shows a sharp increase (3-7 times more), coinciding with a vast increase in the volume of the hepatopancreas. The late larvae also retain food in their guts for longer periods of time (2-3 times longer than early larvae) providing more

chance to digest and assimilate more energy and nutrients from the diets. These larvae can survive on formulated diets from Z5-6 until the metamorphosis, but with a significantly slower growth, lower survival and 1-3 days delay in larval development. It appears that the caridean larvae cannot easily obtain available protein from the artificial diets used in the present study.

Although several authors argue that exogenous enzymes from the live prey may contribute to digestion process of fish and crustacean larvae (Munilla-Moran et al., 1990; Jones et al., 1993), this contribution is insignificant in *M. rosenbergii* larvae fed *Artemia* throughout larval stages (Kamarudin et al., 1994). It is suggested that improvement of current microencapsulated diets for decapod crustacean larvae will depend on the use of partially digested ingredients, inclusion of gut enzyme stimulants or digestive enzymes, while stability of the particles must also be maintained. Although inclusion of digestive enzymes may provide benefits, it is unlikely that this will be feasible as the food particles with enzymes will be highly unstable and uneconomical (Le Vay, 1994). Promising results have been shown in the present study in that the inclusion of alga as a gut enzyme stimulant into the current microcapsules enhances trypsin activity of *P. indicus* at PZ1 stage. Further studies are, however, urgently needed to conclude if alga included diets will also improve growth and survival of penaeid larvae at protozoeal stages. It may be argued that addition of cultured algae into formulated diets will be costly. Yet, several authors have claimed that use of frozen or dried algae as sole diets may be economical in replacing live algae for the culture of a range of marine organisms including penaeids (Aujero and Millamena, 1981; Laing and Gil Verdugo, 1991). The latter report that it is possible to obtain very high algal cell concentrations by using sugars as energy sources instead of light to produce dried marine microalgae by spray-drying technique at commercial levels. It is known that there are commercially available dried algal ingredients, such as *Tet-*

raselmis sp., and *Spirulina* sp., These ingredients should be tested on growth, survival and trypsin activity of penaeid larvae (Le Vay, 1994). Since proteolytic enzyme activity in early caridean larvae is low and stimulation of production levels does not appear to be possible, it is suggested that pre-digested ingredients and/or digestive enzymes should be considered for inclusion into artificial diets for rearing carnivorous larval species such as *P. elegans* and *M. rosenbergii* and possibly *Homarus* sp.

For the first time, Chapter 7 and results of previous chapters provide an opportunity to compare feeding and digestive enzyme strategies of various decapod crustacean larvae. Results indicate that herbivorous decapod larvae, such as penaeids, employ different feeding mechanisms to maximise their chance to obtain enough food when they live in the plankton in comparison to carnivorous decapod larvae. Their digestive system has evolved to enable them to graze on phytoplankton during protozoeal stages. As the guts of herbivorous penaeid larvae lack masticatory capability or a filter apparatus, they maximise energy uptake from less digestible microalgae by secreting high level of digestive enzymes from the AMD (until the HP takes over at mysis stages) and short food retention time (Jones and Kurmaly, 1987; Abubakr and Jones, 1992). Present results with *P. indicus* confirm results with other penaeid species such as *P. monodon* (Kumlu et al., 1992) and *P. japonicus* (Kamarudin, 1992; Le Vay et al., 1993) and demonstrate that penaeid larvae produce higher levels of trypsin activity at herbivoral stages than mysis and early PL stages when they are omnivorous or carnivorous.

Unlike herbivorous larvae, carnivorous decapods such as caridean (*P. elegans* and *M. rosenbergii*), and nephropid (*N. norvegicus* and *H. gammarus*) larvae are large but have limited enzymatic capabilities, due to a lack of secretory AMD and underdeveloped HP. Hence, they require easily digestible and assimilatable prey and retain the food in the gut for longer periods of

time to satisfy the energy need for their large bodies. In contrast to herbivorous penaeids, these larvae are capable of storing lipids as energy reserves to withstand long starvation periods when the prey is scarce or unavailable (Anger and Dawirs, 1981; Sasaki et al., 1986). Results of the present study have shown that specific larval trypsin activities of several species of carnivorous decapods (i.e. *P. elegans*, *M. rosenbergii*, *H. gammarus* and *N. norvegicus*) are similar and approximately 12-17 times lower than herbivorous penaeid larvae and copepods (*T. longicornis* and *C. typicus*). Omnivorous decapod larvae, such as mysis penaeid larvae or crab larvae (*C. maenas*), show an intermediate level of trypsin activity between carnivorous and herbivorous larvae. Although these omnivorous larvae may prefer animal prey, they are able to switch between phytoplankton and zooplankton during larval development. This flexibility gives a great chance of survival to these opportunistic larvae in the marine planktonic communities. The present results indicate that, to date, decapod crustacean larvae with only high trypsin activities are successfully cultured totally on artificial diets and that measurement of trypsin activity appears to be a useful tool to decide whether larvae of a decapod species can be reared on formulated diets.

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OPTIMUM REARING CONDITIONS FOR *PENAEUS INDICUS* LARVAE REARED IN THE LABORATORY

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For penaeid larval nutritional research it is essential to obtain supplies of disease free larvae at regular intervals throughout the year. *Penaeus indicus* originating from the Far East have now been reared through several generations in recirculation systems in Europe. Present work describes the optimum rearing conditions for larvae originating from this disease free stock.

Five different concentration levels (from 10 cells to 50 cells μl^{-1}) of three algal species, *Tetraselmis chuii*, *Rhinomonas reticulata*, and *Skeletonema costatum* were tested singly and in combinations as food for the larvae. The best survival (77%) and growth (5.42mm) at PL1 were obtained by feeding the larvae on a mixed 60 cells μl^{-1} algal diet (25 cells *Tetraselmis*+35 cells *Skeletonema*) plus 5 *Artemia salina* ml^{-1} after PZ3. None of the algal species fed singly gave satisfactory survival and growth rates within the range of algal concentrations tested. However, results clearly show that *Rhinomonas* either alone or in combinations is not a suitable algal species for *P. indicus* larvae.

In a second trial, higher concentrations of *Skeletonema* and *Tetraselmis*+*Skeletonema* (from 50 to 80 cells μl^{-1}) were tested. The results indicate that *P. indicus* larvae need between 50 and 70 cells μl^{-1} of these algal species throughout larval stages and 5 *Artemia* ml^{-1} after stage PZ3.

The effect of 4 different salinities (from 20ppt to 35ppt) on the survival and growth of the larvae from PZ1 to PL1 were also examined. Over 90% survival until PL2 was obtained when the larvae were subjected to 25ppt and 60 cells μl^{-1} *Tetraselmis*+*Skeletonema*. Moreover, the larvae surprisingly passed through 3 zoeal and 3 mysis stages to reach postlarvae within only 6 days. Local seawater at 35ppt salinity gave a significantly lower survival and growth rate.

These trials demonstrate that *P. indicus* larvae can be reared from PZ1 to PL1 within 6 days with over 90% survival in water at 25ppt salinity and at 27-28°C by feeding a combination of *T. chuii*+*S. costatum* plus *Artemia* after PZ3.

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RECENT ADVANCES IN THE DEVELOPMENT OF MICROENCAPSULATED DIETS FOR SHRIMP LARVAL CULTURE

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ABSTRACT

Among a wide range of artificial diets developed to replace live feeds, micro-encapsulated diets have proved to be the most successful for partial or complete replacement in rearing penaeid shrimp larvae. However, growth of larvae fed live diets is still generally superior to larvae fed solely on encapsulated diets. For caridean larval culture, no successful complete replacement of live diets is currently possible. Digestive capability of these larvae appears to be limited due to their poorly developed gut. While inclusion of a small amount of algae into microencapsulated diets induces digestive enzymes and improves growth rates of penaeid larvae, incorporation of exogenous enzymes may be necessary to rear carnivorous caridean larvae at least during their early stages.

1. INTRODUCTION

A vast number of hatcheries have been established to meet the demand for seed by the shrimp industry over the last few decades. These hatcheries conventionally rear penaeid shrimp larvae on microalgae (diatoms, flagellates, etc.) during zoeal, and zooplankton (*Artemia*, rotifers) during later stages. Production of live diets at a commercial scale is complicated, expensive, and unreliable in supply and nutritional value [1,2]. Although *Artemia* is the most practical animal prey, limited resources, high cost of its cysts, and nutritional variability are disadvantages of this live feed source. Therefore, a wide range of artificial diets have been manufactured in an attempt to completely or partially replace live diets in culture of these larvae. Although complete replacement of live diets has been limited in success, partial replacement is already routinely used in many hatcheries. In this paper, we review the latest developments in microencapsulated diets as a result of studies into the digestive physiology of penaeid and caridean shrimp larvae.

2. FEEDING AND DIGESTION

Penaeid shrimp larvae obtain their food by filtering microalgae from the water at protozoeal stages, and capturing zooplankton at mysis and postlarval stages. Caridean larvae, however, consume zooplankton directly 24-36h after hatching. Use of mixed algal diets for penaeid shrimp larvae always gives superior survival, growth and development to single algal species [3] due to their more balanced nutrient content. Cell size of microalgae used to feed early shrimp larvae are generally between 5-20 μm in diameter, whereas the

size range of animal prey ranges from 70 to 500 µm. *Artemia salina* nauplii are the only realistic live prey for both penaeid and caridean larvae such as *M. rosenbergii* until their early postlarval stages.

An appropriate artificial diet can only be developed when the digestive physiology and feeding behaviour of an organism are fully understood. Recent studies on the digestive system of penaeid and caridean shrimp larvae [4] has contributed towards the understanding of the digestive physiology of these larvae. Mandibles of shrimp larvae are able to crush and masticate food particles before the ingestion. The larvae are chance encounter feeders and need a high density of food particles in suspension at all times. Once contact is made the chemical and mechanical cues become important, and the larvae either consume or reject the particles. Penaeid shrimp larvae are less selective than caridean and homarid larvae, accepting inert particles even at mysis stages unless they contain toxic substances [5]. The digestive system of penaeid shrimp larvae is very simple and lacks a gastric mill, and filter apparatus during herbivorous stages. Hence, digestion is mainly conducted by enzymes, released mainly from the AMD (anterior midgut diverticulae) and the hepatopancreas. At mysis stages, the teeth of the gastric mill are fully developed and, thus, the larvae become increasingly carnivorous, retaining food longer and assimilating a higher percentage of energy from their prey. Although penaeid larvae exhibit low assimilation efficiency during herbivoral stages [6], their survival on microalgae and artificial diets is thought to be due to their short gastroevacuation time (GET) and a sufficient amount of digestive enzymes produced by the AMD [4]. Caridean shrimp larvae, such as *Palaemon elegans* and *M. rosenbergii*, however, have very limited digestive capabilities during early stages due to an underdeveloped hepatopancreas, but enzyme activities increase sharply at stage Z5-6 when they are able to survive on artificial diets alone. These larvae appear to rely on prey autolysis for digestion especially during their early stages. Lobster larvae (*Homarus gammarus*) display a high assimilation efficiency on live diets, but cannot reach metamorphosis [5] on encapsulated diets due to their low level of enzyme secretion (Kumlu, unpublished) and long GET. Tissue trypsin activities (IU μg^{-1} larvae body dry weight) for caridean and lobster larvae are very similar and much lower in comparison to that of penaeid larvae.

3. ARTIFICIAL DIETS

Different processing techniques have been employed to produce artificial particles in dehydrated forms as food for aquatic animals. All these processing methods were extensively reviewed by Langdon et al., (1985). Since then, spray drying techniques, which involve spraying a homogenised mixture of ingredients into hot air to form heat sealed and water-stable capsules, have increasingly been used to produce diets for penaeid larvae. Whichever processing method is used, the artificial diet must satisfy the same parameters: acceptability, digestibility, stability, adequate nutritional content, cost-

effectiveness, and storage. The most commonly used artificial diets to culture shrimp larvae are microbound and microencapsulated diets [4].

3.1. Microbound Diets

Microparticulated (microbound) diets are inexpensive, easy to produce and are reported to be used successfully in laboratory and hatcheries [2]. These diets are produced by mixing the nutritional ingredients thoroughly with binders (carboxymethyl cellulose, calcium alginate, carrageenan, agar or gelatine). The mixture is then oven- or freeze-dried, ground and finally sieved through appropriate sizes. They exhibit poor stability in water causing not only water pollution and bacterial built-up, but also they may become deficient due to nutrient leach loss.

3.2. Microencapsulated Diets

The microencapsulation technique was first modified from Chang et al., (1966) to deliver nutrients in a protein and nylon cross-linked membrane to prevent nutrient loss through leaching and used to identify specific nutritional requirements of aquatic organisms. Further development of the technique resulted in the production of only cross-linked protein walled capsules, capable to withstand drying, which have been used extensively in commercial hatcheries [4]. These encapsulated diets promote good survival, but slower growth rate and development in penaeid and prawn larvae in comparison to live diets. Growth and survival equivalent to live diet has been reported for *P. monodon* [8] and for *P. indicus* (Kumlu, unpublished) when a small amount of live or frozen algae (10-15 cells μl^{-1}) was used as a supplemental co-feed with microencapsulated diets. Recent trials show that this supplement of algae significantly induces trypsin activity and hence improves growth and survival in *Penaeus indicus* larvae. Furthermore, preliminary trials indicate that algae incorporated into microencapsulated diets also produces the same effect. It is hoped that these developments will lead to complete replacement of live foods in penaeid culture.

Complete replacement of live diets by artificial diets to rear caridean shrimp and homarid larvae is not currently possible. Despite considerable efforts to develop an adequate artificial diet as a substitute and/or supplement, hatchery production of *M. rosenbergii* still relies heavily on live *Artemia* at least during its early stages. Live *Artemia* was replaced completely in *M. rosenbergii* culture from stage Z6 to Z11 with a microencapsulated diet designed for penaeid larvae [9], and recently *P. elegans* larvae have been reared from stage Z5-6 to PL1 solely on a microencapsulated diet. The inability of the early larvae of these species to survive on artificial diets is attributed to their low digestive enzyme activities. Exogenous enzymes originating from animal food sources and prey are thought to contribute to the digestion processes of both shrimp larvae. For these larvae, it will be necessary to incorporate enzymes within the encapsulated diet to assist in digestion or utilise pre-digested ingredients.

6. CONCLUSIONS

Current microencapsulated diets for penaeid shrimp larvae can be improved by adding gut enzyme stimulants such as microalgae into the diets. Inclusion of exogenous digestive enzymes and the use of partially digested ingredients may overcome the digestibility problem of artificial diets for caridean and other carnivorous larvae.

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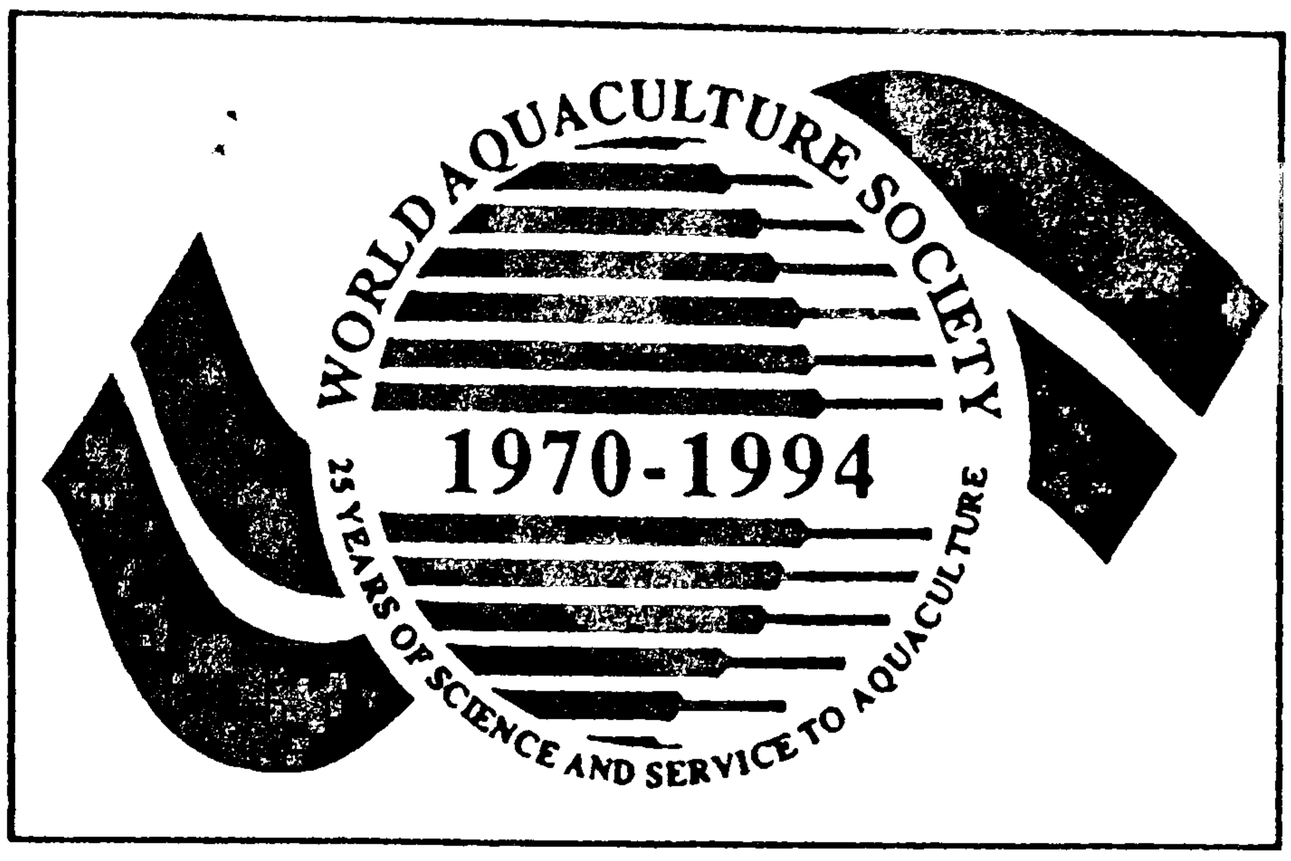

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Growth and Survival of *Penaeus indicus* Larvae Fed on the Nematode *Panagrellus redivivus*.

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Panagrellus redivivus is a free living nematode species found in soil and fermenting substrates. It has shown potential as an *Artemia* replacement feed during the later larval culture stages in some penaeid species.

The purpose of this study was to demonstrate the potential of *P. redivivus* as a total algal/*Artemia* replacement feed during the larval culture of *P. indicus*.

Nematodes were grown in 250 ml baffled flasks containing 50 ml of liquid medium comprised of kidney, vegetable oil and yeast extract. Cultures were inoculated with *Escherichia coli* 24 h previously. All cultures were monoxenic and grown at 22 °C. Nematodes were harvested when maximum populations occurred at approximately 150,000 nematodes per ml. The nematodes were extracted from the media and packed in 15x18 cm high density polyethylene bags containing 2 g of foam blocks and 20 ml of water.

Initially, the *P. indicus* larvae were fed on algae until the zoea 2/zoea 3 (Z1/Z3) stage. At Z2/Z3 the larvae were stocked in 2-l round bottom flasks at a density of 75/l. Control larvae were fed 25 cells/µl/day of both *Tetraselmis chuii* and *Skeletonema costatum* until mysis 1 (M1) when *Artemia* were fed at 5/ml in conjunction with the algae. Algal feeds ceased at M2. Nematodes (with no algal co-feed) were offered at 15, 30, 45 and 60/ml. Fifty percent water exchanges were performed every two days when the larvae were measured and staged. Total water exchanges were performed on alternate days when larval survival was also measured.

To the first postlarval (PL1) stage, the nematode fed larvae demonstrated equivalent growth rates to the controls irrespective of feeding level. Survival was superior to the algae/*Artemia* fed larvae. Water quality in the nematode fed flasks was high, and there was no larval fouling. Preliminary trials have also indicated that *P. redivivus* may be used as a total algal replacement feed from Z1. Trials are continuing to determine optimum nematode size range and feeding rates, lipid and pigment enrichment levels for *P. indicus* larvae from Z1 to PL1.

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Growth and Survival of *Penaeus indicus* Larvae Fed on the Nematode *Panagrellus redivivus* Enriched with Astaxanthin and Various Marine Lipids.

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The nematode *Panagrellus redivivus* promoted good growth and survival of *Penaeus indicus* larvae through to the postlarval stage with no algal/*Artemia* co-feeds. Nematodes may be used as a total algal and *Artemia* replacement feed for the successful culture of *P. indicus* giving growth and survival at least equivalent to that obtained on standard live feeds. In the present study the performance of *P. indicus* larvae fed on nematodes enriched with a variety of marine lipids and astaxanthin (F. Hoffmann-La Roche Ltd.) was monitored. Optimum nematode size and feeding rates were also determined. During zoeal growth, the control larvae receiving only algae and the larvae receiving capelin oil (CAP) enriched nematodes showed superior growth compared to those treatments receiving nematodes enriched with cod liver (COD) or 'Marilla' (MAR) oils. The latter treatments showed equivalent growth. From mysis 1 the controls and larvae receiving the MAR enriched nematodes grew at similar rates and were both significantly faster than all other treatments. Overall the non-enriched (NEN) nematodes resulted in the slowest growth. There was no significant difference in survival between any of the enriched nematode treatments, but all gave superior survival compared to NEN and the control treatments. Nematodes enriched with astaxanthin and code liver oil resulted in significantly faster growth during zoeal stages only. However, this live feed did result in the best overall performance compared to those larvae receiving placebo astaxanthin and/or lipid enriched nematodes. Astaxanthin enriched nematodes did not influence larval survival but pigmentation of the postlarvae was greatly enhanced. Growth and survival of *P. indicus* larvae were not influenced by the size range of nematodes offered.

Role of Microalgae as a Gut Enzyme Stimulant in Rearing *Penaeus indicus* Larvae on Microencapsulated Diets. Metin Kumlu*, and David A. Jones. School of Ocean Sciences, University of Wales Bangor, Gwynedd, Menai Bridge, LL59 5EY, UK.

Recent investigations have shown that when formulated diets are fed to penaeid larvae in conjunction with algae, an improvement in survival and growth may be achieved. In this study, the effects of frozen mixed algae (15 cells μl^{-1} of *Tetraselmis chuii* and *Skeletonema costatum*, 1:2), as a supplement to experimental microen-capsulated (MED) diets (8 mg l^{-1} day^{-1}), on growth, survival and trypsin-like activity in *P. indicus* larvae were examined. Trypsin responses in larvae fed MED's containing microalgal material were also investigated. PZ1 larvae were stocked at a density of 200 individuals in 2-l round bottom glass flasks in filtered (0.2 µm) and UV-treated sea water at 28 °C and 25 ppt salinity. Survival to PZ3/M1 was highest in larvae fed either live mixed algae (91%) or MED plus frozen algae (85-92 %), and lowest when MED was fed alone (46-55 %). The mixture of MED with the algal co-feed supported significantly better growth rates (0.55-0.56 mm day^{-1}) than MED alone (0.42-0.46 mm day^{-1}). Larvae fed MED with the algal co-feed demonstrated a significantly higher tissue trypsin activity throughout the herbivorous larval stages in comparison to MED fed alone ($P<0.05$). These significant improvements in larval growth and survival are likely to be due to higher larval digestive enzyme activities and possibly more efficient digestion by the larvae. When a MED containing freeze dried algal material (23% v/v, *Rhinomonas reticulata*) was fed to *P. indicus* larvae, trypsin activity in PZ2 larvae was equal to that larvae fed live algae. Larvae reared on the MED alone exhibited significantly lower trypsin activity compared to the other diets ($P<0.01$). Hence, algal substances triggering digestive enzyme production are retained within the microcapsules. Present results suggest that inclusion of microalga in formulated diets can act as a gut enzyme stimulant in penaeid larvae with the potential to improve survival and growth particularly during protozoeal stages.

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