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SUMMARY

The effects of live foods and microencapsulated diets on growth, survival, body composition and digestive physiology of fish (mirror carp, Cyprinus carpio) and penaeid shrimp (Penaeus japonicus) larvae were examined.

Raising the lipid content of a microencapsulated enrichment diet from 18.3% to 33.6% improved accumulation of highly unsaturated fatty acids (HUFA) and total lipid in rotifers. Lipids in rotifers were assimilated by carp larvae, as demonstrated by improved growth and accumulation of HUFA in larvae reared on rotifers pre-fed the "high-lipid" diet. Poor diet digestibility coupled with low ingestion rates and low digestive enzyme activity in larvae were identified as contributing to the failure of a microencapsulated diet to support good growth and survival when fed directly to first-feeding carp. Preliminary testing indicates that digestibility of microencapsulated diets for fish larvae can be improved by inclusion of pancreatin.

Experiments with Penaeus japonicus indicate that dietary requirements for protein, energy and HUFA by penaeid larvae are lower than previously thought. Foods of low protein content (Chaetoceros gracilis) or low digestibility (microencapsulated diet) elicited high trypsin activity in mysis larvae, compared to those fed Artemia. Larvae receiving both C. gracilis and Artemia exhibited an intermediate level of trypsin activity - which may explain the superior growth and protein retention in postlarvae reared on the mixed regime. A microencapsulated diet did not support growth and carbon and nitrogen retention equivalent to that in larvae fed live food, despite the strong trypsin response in mysis larvae. When used together with a low density of C. gracilis the microencapsulated diet produced postlarvae of the same size and tissue composition as those reared using live food. It is suggested that the algal co-feed may enhance growth through stimulation of trypsin secretion, supply of extra digestible nutrients or provision of unidentified specific growth enhancing factor(s).

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ACCOMPANYING MATERIAL

Jones, D.A., Kamarudin, M.S. and Le Vay , L. (1993) The potential for replacement of live feeds in larval culture. J. World Aquacult. Soc. 24: 199-210.

Jones, D.A., Le Vay, L. and Kamarudin, M.S. (1993). Feeding and nutritional requirements of penaeid shrimp larvae. In: Caldéron, J. and Sandoval, V. (editors), Mems. 1er congreso ecuatoriano de acuicultura. CENAIM, San Pedro de Manglaralto, Ecuador. pp. 45-52.

Kumlu, M., Le Vay, L. and Jones, D.A. (in press) Recent advances in the development of microencapsulated diets for shrimp culture. Proc. 9th Int. Microencapsulation Symp., Ankara, Sept. 1993.

Le Vay, L., Jones, D.A. and Guzmán, J. (in press). Microencapsulated particles as feeds for fish larvae: problems and prospects. Proc. 9th Int. Microencapsulation Symp., Ankara, Turkey, Sept. 1993.

Le Vay, L., Rodríguez, A., Kamarudin, M.S. and Jones, D.A. (1993) Influence of live and artificial diets on tissue composition and trypsin activity in Penaeus japonicus larvae. Aquaculture 118: 287-297.

Le Vay, L., Rodríguez, A., Mourente, G. and Jones, D.A. (1993). Influence of diet on digestive activity and growth of Penaeus japonicus larvae - implications for nutritional studies. In; Carillo, M., Dahle, L., Morales, J., Sorgeloos, P., Svennevig, N. and Wyban, J. (editors) Europ. Aquacult. Soc. Spec. Pub. 19. pp 145.

Rodríguez, A., Le Vay, L., Mourente, G. and Jones, D.A. (1994). Biochemical composition and digestive enzyme activity in larvae and post-larvae of Penaeus japonicus (Bate), during herbivorous and carnivorous feeding. Marine Biology 118: 45-53.

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Chapter 1.

GENERAL INTRODUCTION

Parts of this chapter have contributed to the publications;

Jones, D.A., Kamarudin, M.S. and Le Vay , L. (1993) The potential for replacement of live feeds in larval culture. J. World Aquacult. Soc. 24: 199-210.

Jones, D.A., Le Vay, L. and Kamarudin, M.S. (1993). Feeding and nutritional requirements of penaeid shrimp larvae. In: Caldéron, J. and Sandoval, V. (editors), Mems. 1er congreso ecuatoriano de acuicultura. CENAIM, San Pedro de Manglaralto, Ecuador. pp. 45-52.

Kumlu, M., Le Vay, L. and Jones, D.A. (in press) Recent advances in the development of microencapsulated diets for shrimp culture. Proc. 9th Int. Microencapsulation Symp., Ankara, Sept. 1993.

The development of commercial culture of crustaceans, fish and bivalves has been largely dependant on the rearing of larvae using live natural feeds. Commercial hatchery rearing of penaeid larvae has followed pioneering methods (e.g. Hudinaga 1942) which require phytoplankton to feed early larval stages with the addition of zooplankton, usually Artemia nauplii, for the later larval stages (Liao 1992, Shigueno 1992, Smith et al. 1992). Rearing of larvae of most marine fish species is dependant on supplying a diet of rotifers for small-mouthed early larvae followed by Artemia nauplii for later stages (Watanabe et al. 1983a). Rotifers, in turn, need to be cultured using phytoplankton (Ito 1960) or bakers' yeast (Hirata and Mori, 1967). Hatchery rearing of bivalve larvae and spat is dependant on a supply of algal feeds of small cell size (Webb and Chu, 1983).

Both algal and rotifer culture are susceptible to collapse, necessitating labour-intensive management and overproduction to ensure adequate supplies. While this is less of a problem in the case of Artemia nauplii, which are available as dry cysts and can be readily hatched, there can also be great variation in the nutritional quality of all live feeds, particularly in their fatty acid content (Sick, 1976, Watanabe et al. 1978, 1980, 1983a,b, Léger et al. 1986). The nutritional quality of rotifers for fish larval culture is dependant on the quality of the alga on which they are reared (Scott and Baynes, 1978, Scott and Middleton 1979), and if cultured with yeast will not be nutritionally suitable for marine larvae without pre-feeding with a source of long-chain polyunsaturated fatty acids (PUFA) (Watanabe et al. 1983a).

Thus the main incentive in the development of artificial diets for the culture of larval stages of aquatic species is the potential for reduction of costs and improvement of reliability in the hatchery production phase of commercial aquaculture. A second area of interest exists in the possible applications of defined diets in the investigation of nutrition and feeding ecology of zooplankton in the natural environment (Jones et al. 1972). The real cost of algal production to support commercial hatcheries is difficult to estimate, as culture conditions vary from country to country. Lee and Wickins (1992) estimate that live feed production represents 40% of post-larval seed production cost and 25% of capital investment in penaeid hatcheries. Langdon et al. (1985) give values of U.S.$18-200 kg^{-1} (dry weight) for live algal production but, if failure occurs at a critical stage, costs in lost production can be much higher. For prawn hatcheries which employ both algal and Artemia culture, considerable savings are already possible by partially replacing these with artificial feeds. If, as seems likely (Ottogalli 1991, 1993), total replacement of algae and Artemia becomes routine for penaeid larval culture, rearing costs will be greatly reduced. Although partial replacement of live feeds is possible for early larvae of many fish species, total replacement from first feeding remains a long-term objective.

However it has been demonstrated for Dicentrarchus labrax that savings of up to 80% in Artemia usage, and consequent substantial reduction in juvenile production cost, may be achieved through use of suitable diets for early weaning at 15-20 days post-hatch, as opposed to current practice of weaning after 40-45 days (Person Le Ruyet et al. 1993). Similarly, Ehrlich et al. (1989) estimate that early weaning of Micropterus dolomieui larvae from Artemia to formulated diets could reduce juvenile production costs by 45%.

Design of artificial larval feeds

The processes used to produce artificial larval diets were reviewed by Langdon et al. (1985) and at that time included microbound particles, encapsulation using coacervation and interfacial polymerisation, and lipid wall capsules. More recently spray-drying and freeze-drying techniques have been developed. Whichever process is used, the diets produced must meet the criteria : Acceptability; particles must be of the correct size for ingestion, available in the water at a similar density to live feed and ingested at a similar rate. Stability; formulated diets must remain stable with minimal leach loss and breakdown until ingested. Digestibility; diets must be as digestible as live prey and readily assimilated. Nutritional content; similar to that of natural prey organisms. Storage; suitable for long term (12 month) storage.

In making microbound particles, gels such as carboxymethyl cellulose, carageenan, agar, calcium alginate or gelatin are used to bind dietary components (Teshima et al. 1982, Kanazawa et al. 1982a, Levine et al. 1983, Langdon et al. 1985). The diet can be oven- or freeze-dried and crumbled to the required size range. This type of diet is relatively simple and inexpensive to produce, but can exhibit poor stability in water causing bacterial growth and poor water quality (Adron et al. 1974, Jones et al. 1979a, Goldblatt et al. 1980.)

Coacervation uses reactions at either a solid:aqueous or aqueous:lipid interface to form a microcapsule wall. The method of Green and Schleicher (1957) has been used to make gelatin-acacia microcapsules (Langdon and Waldock 1981). Coacervation has also been used to produce microencapsulated diets with ethyl-cellulose (Langdon and Waldock 1981) and zein-alcohol combinations. However, the use of an aqueous phase can result in loss of water-miscible components, and the process is most effective in encapsulation of lipid-based diets (Langdon 1980, Landgon and Waldock, 1981, Chu et al. 1982).

Lipid walled microcapsules are formed by emulsion of an aqueous phase within a lipid phase, followed by secondary emulsion within a futher aqueous phase to form lipid particles containing the original aqueous emulsion (Langon 1983, Langdon and Siegfried 1984). Such diets offer the advantage of high retention of water soluble

components, such as vitamins, but have not been developed for large-scale use.

Microencapsulation by interfacial polymerisation of dietary protein was developed from a method described by Chang et al. (1966) in which protein and 6,10, nylon were cross-linked to form the capsule wall. Modification of this method can produce nylon-protein walled capsules suitable for rearing filter-feeding stages of marine crustacea (Jones et al. 1974, Jones et al. 1976). Coagulation of the inner liquid phase by heat treatment produces semi-solid microcapsules which are suitable for ingestion by raptorially feeding decapod larvae (Moller et al., 1979). However, nylon-protein capsules are thin-walled and do not withstand drying (Jones et al. 1987) making them unsuitable for large-scale application. Development of a method of cross-linking dietary protein, without use of nylon, to produce protein-walled capsules capable of withstanding drying led to the first successful commercial-scale hatchery use of microencapsulated diets in rearing penaeid larvae (Jones et al. 1987, British patents; 79437454, 2103568). In recent years, this method of production of penaeid larval diets has been superceded by the use of spray-drying to produce encapsulated feeds.

Use of artificial diets in penaeid larval culture

The development of artificial feeds for penaeid larvae (Table 1) has led to the availability and extensive use of a range of products in commercial hatcheries. Both microparticulate and microencapsulated diets are used throughout Asia and in Ecuador (Forbes 1992, Hirono and Leslie 1992, Liao et al. 1992, Shigueno 1992). Generally, artificial diets are used as a partial replacement for live feeds, though total replacement using encapsulated feeds has been demonstrated both at the laboratory and hatchery level (Jones, et al., 1987) and is now routinely practised in some commercial hatchery systems (Ottagalli 1991, 1993). Several authors have demonstrated successful partial replacement of live feeds with microparticulate feeds (Table 1), and Japanese and Taiwanese penaeid larval culture systems rely on a wide range of different compounded diets. However, it has been demonstrated (Liao 1988, Amjad et al. 1992) that the lack of water stability in most microparticulate diets leads to rapid leaching, bacterial build-up and water pollution.

Experience with several species using both microparticulate and microencapsulated diets has shown that while survival equivalent to live feeds may be achieved, growth is usually slower when using artificial diets (Kanazawa et al. 1982, Jones et al. 1987, Galgani and Aquacop 1988, Kurmaly et al. 1989a). Growth equivalent to that of larvae reared on live feeds can be achieved using either a low density algal supplement with micro-encapsulated artificial diets (Amjad et al. 1992, Kamarudin 1992) or the complete replacement of algae through the protozoeal stages

followed by an addition of Artemia nauplii during the mysis period (Ottagalli, 1993). Amjad et al. (1992) found that the addition of only 10 algal cells.μl^{-1} to the culture water containing microcapsules during protozoeal stages was sufficient to produce growth similar to live fed controls. Algal homogenates and extracts taken from frozen algae added at an equivalent concentration (10 cells μl^{-1}) also produced this growth improvement.

Kurmaly et al. (1989b) report similar assimilation efficiencies by P.monodon larvae fed on live algal feeds or encapsulated diets. The suggestion that the large anterior midgut diverticulae found in P.monodon protozoea are an adaptation to produce sufficient enzymes for the digestion of phytoplankton (Jones and Kurmaly, 1987) has now been confirmed for this species (Abubakr and Jones, 1992), and P.setiferus (Lovett and Felder 1989, 1990a,b), and may explain the ability of penaeid larvae to effective assimilate of artificial diets (Kurmaly et al. 1989b).

Use of artificial diets in culture of other crustacean larvae

In contrast to experience with penaeids, there is as yet no successful replacement for live feeds in caridean prawn or homarid lobster larval culture (Table 1). Macrobrachium rosenbergii hatchery culture still relies primarily upon Artemia nauplii. Partial replacement of live feeds with modifications of the egg custard diet developed by Ling (1969) is now routine, but first feeding larvae must be fed Artemia nauplii. Deru (1990) demonstrated that it is possible to totally replace Artemia with an encapsulated diet, but only from larval stage VI onwards.

Larval stages of Macrobrachium and Homarus display encounter feeding and may accept inert particles, although ingestion usually only occurs if particles are nutritious. However, Kurmaly et al. (1990) have shown that lobster larvae use dietary conditioning behaviour to reject artificial diets unless attractants are regularly alternated. In their study, a wide range of microparticulate and encapsulated diets have been used in an attempt to replace live or natural (frozen mysids) diets for lobster (Homarus gammarus) larvae. Despite the range of dietary materials presented, larvae did not moult beyond stage III on artificial diets. High assimilation efficiencies for Homarus larvae were observed only when they were fed live diets, and Kurmaly et al. (1990) concluded that low enzyme secretion levels together with a long gut retention time precludes total replacement of live diets at present.

Use of artifical diets in culture of fish larvae

As can be seen in Table 2, few fish species have been successfully reared from first feeding exclusively on artificial diets, and in most cases success at the experimental level has yet to be reproduced on a commercial scale. While Table 2 shows that most fish larvae accept inert particles, there is considerable literature on their feeding

behaviour which suggests that at least some species (e.g. Clupea harengus) display visual prey selection triggered by prey movement (Rosenthal and Hempel, 1970; Fox, 1990). In addition, taste and texture of particles once taken into the mouth may elicit rejection (Jones et al., 1984).

As larval fish are carnivorous at first feeding, it might be expected that they will display difficulties in digesting artificial feeds similar to those seen in carnivorous crustacean larvae. The degree of success with which diets may be used appears to depend, to a large extent, on the mode of development of gut function for each species.

In general, freshwater larvae are fairly advanced at hatching and can adapt to dry feeds relatively easily. This is true particularly for the salmonids (12-25mm at hatching) which possess a functional stomach at first feeding and do not require live prey at this stage. Larvae of fish which remain stomachless throughout life, such as cyprinids, are reported to show a rapid development of trypsin-like protease activity during early life (Kawai and Ikeda 1973), and can adapt relatively easily to artificial diets (Table 2). Amongst the freshwater fish, the most promising results have been achieved with coregonid larvae which in many experimental studies have been reared exclusively on dry diets (reviewed by Champigneuille 1988). It is now possible to mass rear Coregonus lavaretus larvae from first feeding on a yeast based dry diet, with good growth and survival (Champigneuille 1988). Other freshwater species which have been reared on artificial diet include Cyprinus carpio (Charlon and Bergot 1884; Charlon et al. 1986) Plecoglossus altivelus (Kanazawa et al. 1985) and Micropterus dolomieui (Ehrlich et al. 1989).

Most marine larvae hatch with small yolk reserves and feed at an early stage (2-3mm length), when they have no functional stomach and possibly poor development of pancreatic enzyme secretion (Beccaria et al. 1991, Boulhic and Gabaudan 1992). Success with total replacement of conventional live feeds for such larvae has been extremely limited due to their inability to digest artificial feeds (Kanazawa et al. , 1982b, Appelbaum, 1985, Walford et al. 1991). The suggestion that the enzymes of prey contribute a large proportion of the proteolytic activity measured in fish larvae (Dabrowski and Glogowski, 1977a) has been supported by Lauf and Hofer (1984) who found lower enzyme levels in larvae fed zooplankton processed to denature contained enzymes. Further support is supplied by Munilla-Moran et al. (1990) who estimate that prey zooplankton make a very significant contribution to the digestive activity in first feeding turbot larvae. These authors also demonstrate that pre-feeding prey organisms (Artemia and copepods) elevates enzyme activity levels within the prey, thus enhancing the exogenous contribution made by the prey to fish larval digestion.

In contrast, Hjelmeland et al. (1988) argue that an exogenous prey enzyme requirement for larval fish is not proven, and cite as evidence experiments in which herring larvae secreted trypsin in response to feeding with inert polystyrene beads (Hjelmeland, 1988). These authors and others (Pedersen et al. 1987, Munilla-Moran and Stark 1989, Rosch and Segner, 1990, Day et al. 1993) provide evidence for de novo enzyme production by fish larvae in response to both live and artificial diets.

Several attempts have been made to supply enzymes in artificial diets to fish larvae in an attempt to improve digestion (Dabrowski and Glogowski 1977b, Dabrowska et al. 1979, Kolkovski et al. 1993). The former authors, working with carp larvae, demonstrated elevated levels of gut enzyme activity, but saw no improvement in growth. The latter authors report at improvement in growth of Sparus aurata larvae fed microdiets containing pancreatin while Kolkovski et al. (1993) found that a similar enzyme inclusion improved protein assimilation by early Sparus aurata larvae. Walford et al. (1991) have suggested that co-feeding live food with microcapsules, so that prey enzymes may assist in their breakdown, can enhance digestion of the artificial diet by Lates calcarifer larvae.

Despite the apparent problems, complete weaning onto artificial diets can be achieved earlier than is currently practised for Dicentrarchus labrax (Person Le Ruyet et al. 1993) and potentially for Solea solea (Appelbaum 1985; Gatesoupe 1983). Also, some early larvae can be successfully fed artificial diets as a partial replacement for, or supplement to, live feeds. In some cases, co-feeding live and artificial diets can produce growth and survival in early larvae superior to that achieved with either live feeds or artificial diets alone. This has been found for Micropterus dolomieui (Ehrlich et al. 1989), Chanos chanos (Marte and Duray 1991), Lates calcarifer (Walford and Lam 1991) and Sciaenops ocellatus (Holt 1991). This form of early weaning may allow a reduced dependence on live feeds, and up to 90% substitution with artificial diets has been achieved for Pagrus major and Paralichthys olivaceus with 10 day old larvae (Kanazawa et al. 1989). Sparus aurata larvae have been reared successfully from first feeding with 50% live feed substitution (Vergara Martin et al. 1990), and up to 80% substitution with microdiets is possible without impairing growth (Kolkovski et al. 1993).

Use of artificial diets in culture of bivalve larvae.

Experimental studies on the application of artificial diets to bivalve rearing are listed in Table 3. Although Crassostrea virginica larvae have been reared through metamorphosis using microencapsulated diets, survival was poor (Chu et al. 1987) and development of effective commercial encapsulated feeds for bivalve larvae

is likely to be restricted by the technical difficulty and cost of production of the small size of particle required. Spray-dried algal products present the best alternative for replacement of live algae in bivalve rearing, although larvae smaller than 150 μm may be unable to ingest spray dried Tetraselmis (Laing et al. 1990). Freeze dried algae have been used to rear Mercenaria mercenaria larvae to metamorphosis (Hidu and Ukeles, 1962), and growth of Tapes philippinarum larvae reared on spray-dried Tetraselmis can be equivalent to that achieved with live Tetraselmis (Laing and Verdugo, 1991). However, in the same study, better growth was achieved using a mixed live algal diet. Growth similar to that on live Tetraselmis has also been achieved for spat of five oyster and clam species when they were fed this alga in a spray dried form (Laing and Verdugo 1991). However, growth was less than that achieved on live Chaetoceros or on live algal mixtures unless a 20% supplement of live Chaetoceros was supplied.

Spat and juvenile oysters and clams accept a wider range of particle sizes than do larvae, thus live replacement feeds with artificial diets have proved more successful. Microbound particles and microcapsules (Langdon and Siegfried, 1984; Laing, 1987) have been shown to support up to 70% of the growth obtained on live algal diets, and when used as partial replacements (50:50), may compensate for nutritional deficiencies present in algal cultures.

Artificial diets have proved effective in the conditioning of broodstock for spawning. Lane (1989) demonstrated that Ostrea edulis fed a 50:50 (w/w) diet of Isochrysis together with microcapsules high in HUFA produced significantly more larvae than algal-fed controls.

Nutritional composition of artificial diets

Only when a water stable formulated artificial diet is accepted, ingested, digested and assimilated at rates comparable to live feeds will it be possible to investigate specific nutritional requirements of larvae. Currently most larval diets are formulated from fish or shellfish meals, cod roe or other natural products and have a broad composition similar to that of live plankton feeds (Table 4).

Recent reviews of the nutrition of bivalves (Webb and Chu, 1983), crustaceans (Kanazawa 1984, 1990), and fish (Cowey et al. 1985, Steffens 1989) provide only limited information on larval nutritional requirements. There is an apparent general requirement for HUFA in marine larvae, reflecting the high levels of n-3 HUFA entering the pelagic food web from phytoplankton.

Marine fish have a highly limited ability to elongate and desaturate fatty acids of the n-3 series to produce 20.5n-3 and 22.6n-3 HUFA (Owen et al. 1975, Cowey et al. 1976a,b). Studies of live feed organisms have shown that it is largely C_{20} n-3

HUFA content which determines their suitability for marine larval fish culture (Fukosho 1977, Scott and Middleton 1979, Kitajima et al. 1979, Watanabe et al. 1978, 1979, 1982, 1983a). Apart from a requirement for n-3 HUFA, particularly 20.5n-3 and 22.6n-3, little else is known of the dietary requirements of marine fish larvae due to the lack of acceptable and digestible defined feeds. However, a possible need for free amino acids, which are present in high concentrations in zooplankton and are readily absorbed by the simple larval gut, has been suggested (Fyhn 1989).

In the case of penaeid larvae, which readily accept and ingest diets, some conclusions as to the nutritional requirements can be drawn. Table 5 summarises available literature on nutrient requirements for penaeid larvae obtained by feeding known levels of nutrients in diets. These values are levels of dietary components which have allowed successful larval culture and do not necessarily reflect optimal requirement levels. Inclusion levels of protein in successful larval diets for P. monodon range from 30%-56% dry weight, demonstrating some correlation with live diets where the same species is reared through protozoeal stages on Skeletonema, Tetraselmis and Rhinomonas which range from 33%-52% protein (Table 1). However, although most authors suggest a minimal protein requirement for P. japonicus larvae of 45% (Table 5) it is common practise to use Chaetoceros gracilis, a species of low protein content as the main algal species for culture of this and other species in many parts of the world (Forbes 1992, Liao 1992, Smith et al. 1992). To date few published studies have been made on the relative digestibility of protein sources used in feeds for larval penaeids, as is becoming routine in the formulation of ongrowing feeds (Akiyama, 1992, Lan and Pan, 1993).

Lipid nutrition of penaeid larvae has received more attention, and a dietary requirement for 20.5n-3 and 22.6n-3 HUFA has been demonstrated using partially defined microencapsulated diets (Jones et al. 1979b). Most algal total lipid levels are in the region of 4%-8% and levels from 4% to 23% have been successful in artificial diets, suggesting that 5%-6% for protozoeal stages and perhaps 6%-12% for mysis stages are adequate. Inclusion of HUFA at the 1%-2% level is indicated, and dietary requirements for cholesterol (0.5%-1%) and phospholipid (3%) have been demonstrated (Table 5).

Kanazawa (1990) has demonstrated a protein sparing effect of carbohydrate in diets for P. japonicus larvae, though there is still speculation as to the availability of algal carbohydrates for digestion by prawn larvae (Lovett and Felder 1989). Kanazawa (1986) has listed the essential vitamins for P. japonicus larvae as a result of feeding trials, and has given recommended inclusion levels for these (Kanazawa 1990). As he points out these can only be regarded as 'safe' levels rather than optimal, as leaching and bacterial contamination prevent more precise measurement to date.

Enrichment of live feeds using artificial diets

Although fish larvae do not generally adapt readily to feeding on artificial diets, primarily due to acceptability and digestibility problems, artificial diets may be fed indirectly to larvae reared on live zooplankton. As described earlier, larvae of many commercially cultured fish species have been found to have dietary requirements for highly unsaturated fatty acids (HUFA), and live zooplankton feeds may provide less than adequate nutrition unless these are pre-fed algae or artificial diets rich in HUFA. Enrichment of live feeds may also have a beneficial effect on growth and survival of crustacean larvae (Léger et al. 1987, Tackaert et al. 1989). Early experimental approaches used emulsions of HUFA-rich oils or enriched yeasts to raise fatty acid levels in rotifers and Artemia nauplii (Table 6) Enrichment of live feeds represents one of the few areas of success in application of artificial diets to larval fish culture, and has led to the development of a range of commercial products used in many hatcheries. Table 6 includes some experimental studies using currently available commercial brands of enrichment diet.

Aims of the present studies

This thesis includes investigations into aspects of the influences of live and artificial foods on growth, survival, body composition and digestive physiology of fish and penaeid shrimp larvae. It is intended that this approach may yield insights into the nutritional requirements of larvae and aid identification of potential areas of improvement to artificial diets. Most of the studies presented here have made use of a well established, commercially available, spray-dried form of encapsulated larval diet - which has been applied in both fish and crustacean culture (as reviewed in this chapter).

Chapter 2 investigates the effects of two microencapsulated diets, of different lipid content, on the lipid composition of rotifers, and evaluates the uptake of elevated lipid levels by fish larvae.

Chapter 3 investigates growth, survival, feeding rates and digestive enzyme activity in mirror carp (Cyprinus carpio) larvae fed live food or a microencapsulated diet. The use of microencapsulated pancreatin to enhance digestion of food particles by fish larvae is demonstrated.

Chapter 4 compares the effects of carnivorous and herbivorous feeding on growth, survival digestive enzyme activity and biochemical composition of Penaeus japonicus larvae.

Chapter 5 examines the influence of live food and microencapsulated diets on growth, survival, digestive enzyme activity and body composition of Penaeus japonicus larvae.

Table 1. Review of studies on replacement of live feeds in larval crustacean culture.

Feed replacement	Result	Author
PENAEID SHRIMP		
P.monodon		
Microcapsules + algae no *Artemia*	9-47% survival to PL7 (20t tank)	Jones *et al*. (1987)
Microcapsules only	3-29% survival to PL7 (1.2t tank)	Jones *et al*. (1987)
Microbound diet	85% survival to M_1	Galgani and Aquacop (1988)
Microbound +algae + *Artemia*	survival and growth less than live-fed controls	Liao *et al*. (1988)
Microcapsules only	51-64% survival less than live-fed controls (2l flasks)	Kurmaly *et al*. (1989a)
Microcapsules only	80% survival, growth lower than live-fed controls (2l flasks)	Amjad *et al*. (1992)
Microcapsules +10 cell μl^{-1} algae	74% survival, growth same as live-fed control (2l flasks)	Amjad *et al*. (1992)
P.stylirostris		
Microcapsules + algae no *Artemia*	65% survival to PL5-7 (25t tank)	Jones *et al*. (1987)
Microcapsules + *Artemia*	growth and survival comparable with live feeds (commercial scale)	Ottogali (1991)
P.japonicus		
Microbound diet	90% survival, growth less than live-fed controls	Kanazawa *et al*. (1982a)
Microbound diet	90% survival, growth same as live-fed (laboratory-scale)	Kanazawa (1985a)

Table 1. (continued)

Feed replacement	Result	Author
P. japonicus		
Microbound diet	75% survival (15t tank)	Kanazawa (1990)
P.vannamei		
Microcapsules+algae + *Artemia* 3-5 ml^{-1}	20% survival to PL5-7 (2t tank)	Jones *et al.* (1987)
Microcapsules+algae no *Artemia*	80% survival to PL5-7 (25t tank)	Jones *et al.* (1987)
Microbound	42% survival to M_1 growth less than with algae	Galgani and Aquacop (1988)
Spray dried algae +*Artemia*	84% survival on 66% replacement of algae	Biedenbach *et al.* (1990)
Microcapsules +*Artemia*	Commercial-scale production	Ottogalli (1993)
P. indicus		
Microbound diet	62% survival to M_1 growth less than with algae	Galgani and Aquacop (1988)
CARIDEAN SHRIMP		
Macrobrachium rosenbergii		
Freeze dried catfish (from stage VI)	11% survival to PL	Sick and Beaty (1975)
Egg custard, fish, shrimp+ *Artemia*	77% survival to postlarva	Ang and Cheah (1986)
Microcapsules from stage VI	84% survival similar to control, growth 1 day slower	Deru (1990)
LOBSTER		
Homarus gammarus		
Microcapsules, microbound	no survival to postlarva	Kurmaly *et al.* (1990)

Table 2. Review of studies in complete replacement of live feeds for first-feeding fish larvae.

Feed replacement	Result	Author
FRESH WATER		
Cyprinus carpio		
Microbound (yeast based)	90% survival, growth comparable with live feeds	Charlon and Bergot (1984)
Microbound (yeast based)	87% survival, growth comparable with live feeds	Charlon *et al*. (1986)
Microbound	90% survival, growth 25-40% of live-fed.	Slaminska and Pryzybyl (1986)
Coregonus lavaretus		
Microbound (yeast based)	90% survival, good growth, mass rearing	Champigneuille (1988) (and see review therein)
Micropterus dolmieui		
Commercial dry diet	26-45% survival, growth better than *Artemia* control	Ehrlich *et al*. (1989)
Morone saxatilis		
Microparticulate	no survival at 20d	Tuncer *et al*. (1990)
Plecoglossus altivelis		
Microcapsules zein coated microbound	poor survival and growth	Kanazawa *et al*. (1982b)
Microbound	Good growth and survival	Kanazawa *et al*. (1985)
MARINE		
Pleuronectes platessa		
Microbound	survival 50% of live-fed control	Adron *et al*. (1974)

Table 2. (continued)

Feed replacement	Result	Author
Solea solea		
Zein-coated	lower survival, growth than live-fed controls	Gatesoupe et al. (1977b)
Microparticulate/ microencapsulated	lower survival and growth than live-fed controls	Appelbaum (1985)
Dicentrarchus labrax		
Zein- coated	lower survival, growth than live-fed controls	Gatesoupe et al. (1977b)
Pagrus major		
Microcapsules zein-coated microbound	Poor survival and growth	Kanazawa et al. (1982b)
Gadus morhua		
Microcapsule	Poor survival and growth	Garatun-Tjeldstø et al. (1989)
Sparus aurata		
Microbound + exogenous enzymes	Survival and growth better than without enzymes, but still poor.	Kolkovski et al. (1993)
Lates calcarifer		
Microcapsules	no survival after 10d	Walford and Lam (1991)
Clupea harengus		
Microcapsules	poor survival and growth	Fox (1990)

Table 3. Review of studies on replacement of live feeds for bivalve molluscs.

Feed replacement	Result	Author
LARVAE		
Crassostrea virginica		
Gelatin-acacia capsules and lipid walled capsules	low survival to metamorphosis	Chu *et al.* (1987)
Mercenaria mercenaria		
Freeze-dried microalgae	Survival to metamorphosis	Hidu and Ukeles (1962)
Tapes philippinarum		
Spray dried microalgae	Growth same as with same single live alga, but not mixed species.	Laing *et al.* (1990)
SPAT/JUVENILES		
Crassostrea virginica		
Microbound and microcapsules	Variable growth, best 73% of control	Langdon and Seigfried (1984)
C. virginica, *C. gigas*, *Ostrea edulis*, *T. semidecussata*, *M. mercenaria*		
Microcapsules	Growth 54-64% compared to live algae.	Laing (1987)
Microcapsules + 15-40% algae	Growth same as live algae control.	Laing (1987)
C. gigas, *O. edulis*, *T. phillipinarum*, *M. mercenaria*		
Spray dried *Tetraselmis*	Growth same as live *Tetraselmis*, lower than live *Chaetoceros* or mixed algae.	Laing and Verdugo (1991)

Table 4. Proximate analyses and HUFA (n-3) levels of some live foods commonly used in larval culture.

Species	Protein	Carbohydrate	Lipid	HUFAn-3*
	(percentages of dry weight)			
Algae				
Rhinomonas reticulata	52	33.7	4.3	19.2 [1]
Tetraselmis chuii	49	24.7	4.3	3.4 [1]
Chaetoceros spp.	7-23	15-19	4.1-8.7	5.1 [2,3]
Skeletonema spp.	33	22.6	8.1	25.8 [1]
Nematodes				
Panagrellus spp.	48-62	-	2.7-15	9-20 [4,5]
Rotifers				
Brachionus plicatilis	32-60	16.5	14-17	3.1 [4,6,7]
Artemia				
Artemia nauplii	51-55	14.7-18	16-19	4-15 [2,4,6]
Encapsulated feeds				
Frippak CAR/CD	52	13-14	12	2

* HUFA as percentage of total fatty acids

[1] Kurmaly et al. (1989a), [2] present study [3] Tobias-Quinitio and Villegas (1982), [4] Leger and Sorgeloos (1992), [5] Rouse et al. (1992), [6] Watanabe et al. (1983a), [7] Ferreiro et al. (1991).

Table 5. Nutrient requirements and dietary levels for penaeid larvae estimated from review of published feed trials.

Nutrient/ Level	Comment	Author
PROTEIN		
P. japonicus		
45-55%	Given 15-25% carbohydrate	Kanazawa (1990)
40%	Better than 50%	Besbes (1987)
P. monodon		
48-52%	Best growth on live diets	Kurmaly *et al.* (1989a)
51-56%	Best growth on encapsulated diets	Kurmaly *et al.* (1989a)
30%	Microbound diet	Khannapa (1979)
23%	PZ_1-M_1 on *Chaetoceros calcitrans*	Tobias-Quinitio and Villegas (1982)
CARBOHYDRATE		
P. japonicus		
15-25%	Given 45-55% protein	Kanazawa (1990)
P. monodon		
24-33%	Best growth on live diets	Kurmaly *et al.* (1989a)
7.5%-18%	Best growth on encapsulated diets	Kurmaly *et al.* (1989a)
LIPID		
P. japonicus		
Dietary requirement for HUFA		Jones *et al.* (1979b)
1% HUFA suggested level		Guillaume (1990)
3% Phospholipid requirement		Kanazawa (1990)
1% cholesterol requirement		Teshima *et al.* (1983)
P. monodon (live feeds)		
4.3% total lipid, 3.4% HUFA (Z_{1-3})		

Table 5. (continued).

16% total lipid, 12.4% HUFA (M_1-PL_1)	Kurmaly et al. (1989a)
P. monodon (encapsulated diets)	
23.5% total lipid, 26.1% HUFA (PZ_{1-3})	
18% total lipid, 12.4% HUFA (M_1-M_3)	Kurmaly et al. (1989a)

VITAMINS

P. japonicus	
Carotene, thiamine, riboflavin,pyroxidin, nicotinic acid, folic acid, biotin, cyanocobalamin, choline, inisitol, ascorbic acid, vitamin D, vitamin E	Kanazawa (1986)

Table 6. Review of experimental studies of enrichment of live zooplankton feeds with artificial feeds/media. (Brand names are italicised).

Rotifers (Brachionus plicatilis)

AUTHOR(S)	METHOD/COMMENT
Imada et al.(1979)	Used bakers' yeast previously cultured in fish oil medium (omega yeast). Increased n-3 HUFA in rotifers to 25% after 6h feeding.
Watanabe et al. (1983b)	HUFA methyl esters + yeast fed directly
Walford and Lam (1987)	*Frippak CAR*. HUFA content of rotifers dependant of HUFA in dietary lipid.
Opstad et al. (1989)	Emulsified fish meal and cod roe. Reared Gadus morhua larvae to metamorphosis.
Budd (1989)	*Frippak CAR*. Effective elevation of HUFA 0.5g/10^6 rotifers gave optimal results.
Rodriguez Rainuzzo et al. (1989)	Fish oil or squid/fish meal emulsion. HUFA HUFA in rotifers dependant on HUFA in feed.
Kissel and Koven (1990)	Enhancing HUFA content of capelin oil by low temp. distillation gave better results than crude oil.
Kjørsvik et al. (1991)	*Selco*. Highest lipid levels in rotifers supported best growth in Scophthalmus maximus larvae, but may hinder protein digestion.
Komis et al. (1991)	*Culture Selco*. Used to maintain rotifer cultures, yields similar to yeast+algae. HUFA up to 40x higher than after secondary enrichment with *Selco*.
Pousao-Ferriera and Soares (1991)	*Frippak Booster* supported better growth in Sparus aurata larvae than *Protein Selco* or fish oil emulsion. Oil emulsion hindered swim-baldder inflation.
Fernández-Reirez et al. (1993)	*Selco*, *Dry Selco*, *Super Selco* improved total lipid content (to 17.5-19.8% of dry weight), *Protein Selco* and *Dry Selco* increases dry weight and energy content.

Table 6 (continued)

Artemia

AUTHOR(S)	METHOD/COMMENT
Watanabe et al. (1978)	HUFA-enriched yeast and HUFA-yeast mixture.
Watanabe et al. (1980)	Omega yeast-enriched nauplii improved growth of Pagrus major larvae.
Gatesoupe and Luquet (1981)	Cod-liver oil, soluble fish protein dry diet improved survival of Dicentrarchus labrax larvae
Sakomoto et al. (1982)	Nylon-protein microcapsules, HUFA accumulated by nauplii.
Jones et al. (1984)	Simple cod and pollock oil extracts improved growth and survival of Pomatoschistus minutus postlarvae.
Léger et al. (1987)	Cod-liver oil coated rice bran enhanced HUFA in poor quality nauplii, supporting good growth in Mysidopsis bahia larvae.
Léger et al. (1986)	*SEC* self emulsifying enrichment diet improved growth and survival of P. stylirostris larvae fed poor quality nauplii.
Dendrinos and Thorpe	HUFA enriched yeast (Candida utilis improved HUFA content of nauplii and growth of Solea solea larvae reared on them.
Walford and Lam (1987)	*Frippak CAR* improved HUFA content.
Grove and Fletcher (1988)	*Frippak CAR* improved growth in carp larvae.
Karavellas (1989)	*Frippak booster*. Improved HUFA and energy content of nauplii, growth of *Solea solea* larvae.
Tackaert et al. (1989)	Enriched naupli improved survival of PL P. monodon, P. japonicus, P. vannamei.
Dhert et al. (1991)	*Selco* improved survival to metamorphosis and stress resistance in Lates calcarifer larvae.

Table 6 (continued)

Artemia

AUTHOR(S)	METHOD/COMMENT
Bisbal and Bengston (1991)	HUFA emulsions, high 20.5n-3 and low 22.6n-3 gave best growth of Paralichthys dentatus larvae.
Dhont et al. (1990)	*Super Selco* used to ongrow Artemia juveniles gave faster and higher HUFA accumulation than scondary enrichment of nauplii.
Howell and Tzoumas (1991)	*Frippak Booster*. Improved poor quality nauplii to support same growth in Solea solea larvae as good nauplii, reduced occurence of abnormal pigmentaton.

Chapter 2

SECONDARY LIPID ENRICHMENT OF ROTIFERS USING SPRAY-DRIED MICROENCAPSULATED DIETS.

Parts of this chapter have contributed to;

Jones, D.A., Le Vay L., Budd, M.D., and Theodoru J. (1991). Modification of rotifers cultured as food for fish larvae using microencapsulated diets. Oral presentation to the Fermentation and Bioprocessing Group, 119th Meeting of Society for General Microbiology, University of Edinburgh, 9th-12th April 1991.

INTRODUCTION

Lipids are the dominant component of energy reserves in pre-feeding fish larvae, (Ehrlich 1974, Love 1980, Kanazawa 1985b), with triacylglycerols probably the main source of energy (Håkason 1989, Tandler et al. 1989). There is also evidence that phospholipids (Kimata 1983), protein (Fyhn et al. 1987) and free amino acids (Fyhn 1989) contribute to the energy requirements of prefeeding larvae. Once feeding begins, dietary protein becomes a more important energy source than lipid (Kaushik and Dabrowski 1983). Supply of adequate dietary lipid has been demonstrated to improve growth in juveniles and adults of a range of fish species through 'sparing' of dietary protein for tissue growth rather than use as an energy source (Lee and Putnam 1973, Sin 1973, Adron et al. 1976, Reinitz et al. 1978). As well as acting as an energy source, there is also a direct requirement for lipids in growth and development of fish, and it is in this role that the fatty acid composition of lipids is particularly important. Starvation studies of larvae of various marine fish species have demonstrated conservation of 20.5n-3 and 22.6n-3 fatty acids, as opposed to n-6 series, n-9 series or shorter chain n-3 fatty acids, indicating a predominantly important function for highly unsaturated fatty acids (Tocher et al. 1985, Fraser et al. 1988, Koven et al. 1989, Tandler et al. 1989). Cellular membranes in fish contain 25% - 80% lipid, predominantly in the form of phospholipids (Henderson and Tocher 1987).

Dietary fatty acids can be incorporated into phospholipids and the degree of unsaturation of such fatty acids may determine membrane fluidity and functional efficiency of membrane-bound enzymes , and adaptation to external environmental parameters, particularly low temperature and high salinity, may be accompanied by accumulation of polyunsaturated fatty acids in phospholipids in membranes (Bell et al. 1986). Highly unsaturated fatty acids of the n-3 series (n-3 HUFA), particularly 22.6n-3, have been identified as important components of neural and retinal tissue in teleost fish (Sastry 1985, Tocher and Harvie 1988, Bell and Tocher 1989, Mourente et al. 1991, Mourente and Tocher 1992), as is the case in mammals (Neuringer et al. 1988) for which dietary deficiency of n-3 HUFA can result in visual impairment (Lamptey and Walker 1976, Neuringer et al. 1984). Although no specific role for 20.5n-3 has been determined in fish, radiolabelling studies suggest that this fatty acid is mainly incorporated into the swim bladder, intestine and gall bladder and secondarily into liver and gill tissue (Kanazawa et al. 1982c).

In common with other vertebrates, fish are able to synthesise the 14C, 16C and 18C saturated fatty acids de novo. Although vertebrates can desaturate these fatty acids to form n-9 series unsaturated fatty acids, they have a dietary requirement for 18.2n-6 and 18.3n-3. In some marine fish species such as Scophthalmus maximus an

inability to elongate and desaturate dietary 18.2n-6 and 18.3n-3 has been identified (Owen et al. 1975). For such species there is a dietary requirement for long chain n-3 HUFA, particularly 20.5n-3 and 22.6n-3, and a lack of these fatty acids can result in supressed growth and deformity (Yone and Fujii 1975, Cowey et al. 1976a). In contrast, some freshwater fish species, such as Oncorhynchus mykiss, Coregonus lavaretus and Cyprinus carpio are able to synthesise 20.5n-3 and 22.6n-3 from dietary 18.3n-3 (Watanabe et al. 1974, Owen et al. 1975, Farkas et al. 1977, Csengeri et al. 1977, Watanabe et al. 1989). However the rate of synthesis is slow and addition of long-chain HUFA to diets has been found to promote growth more effectively than addition of 18.3n-3 (Takeuchi and Watanabe 1976, 1977, Watanabe et al. 1989).

Since the development of technology for culture of the rotifer Brachionus plicatilis as food for first feeding fish larvae (Ito 1960), bakers yeast, Saccharomyces cerevisiae, has become the standard food for mass rotifer cultures (Hirata and Mori 1967). Brachionus plicatilis has only a limited ability to synthesise n-3 HUFA (Lubzens et al. 1985) and hence HUFA content of rotifers used in fish culture has been found to be largely dependant on the food on which they are grown (Scott and Middleton 1979). Marine fish larvae reared using rotifers which lack 20.5n-3 and 22.6n-3 have been found to exhibit poor growth and high mortality (Owen et al. 1975, Howell 1979, Scott and Middleton 1979, Watabane et al. 1983a). As bakers yeast usually contains little or no 20.5n-3 and 22.6n-3, rotifers grown exclusively using this food are unsuitable for feeding to marine fish larvae (Watanabe et al. 1983a). The n-3 HUFA content of yeast-reared rotifers may be improved by secondary feeding with a suitable algal species, or a HUFA-rich emulsion or microparticulate diet (see Table 6, Chapter 1). Previous studies have shown that nylon-protein and protein cross-linked microencapsulated diets can be used in lipid enrichment of rotifers. (Sakomoto et al. 1982, Walford and Lam 1987). More recently, Budd (1989) has demonstrated that a spray-dried microencapsulated diet can also be effective in improving the fatty acid composition of rotifers.

This aims of the present study are;

1. To evaluate the effects of two spray-dried microencapsulated diets, differing in total lipid content, on the fatty acid composition and total lipid content of the rotifer Brachionus plicatilis.

2. To determine optimal enrichment duration and feeding levels for the enrichment diets.

3. To examine the effect of enrichment on rotifer population density.

4. To demonstrate the effective transfer of lipid from enriched rotifers to fish larvae, using mirror carp, Cyprinus carpio, larvae as a model.

METHODS

Algal culture

Rhinomonas reticulata was grown in semi-continuous batch culture in square-sided polythene carboys (25l capacity). These containers are convenient for culturing large amounts of algae, and can be steam cleaned and reused. Up to six cultures were maintained simultaneously, illuminated by four 80 W warm-white flourescent lights at ambient laboratory temperatures (19 - 23 ^{o}C). All algal cultures were in Conway medium made up with 0.2 μm filtered, U.V. irradiated seawater, salinity 32 ^{o}/oo (Laing 1991). Strong aeration was supplied, unfiltered, from the main laboratory compressed air system though polythene tubing. Cultures were initiated with 3.5 l of Conway medium innoculated with 1.5 l of Rhinomanas reticulata culture from the algal culture collection, School of Ocean Sciences. 5l of Conway medium was added daily until culture volume reached 20l. Cultures were then allowed to grow until cell densities reached 1000 cell.μl^{-1}. Thereafter 5 l of each culture was harvested daily, and replaced with fresh Conway medium. Using this method stable cultures were maintained for up to 4 weeks. After harvesting, algal cells were concentrated by centifugation at 3000 r.p.m. for 10 min (MSE Coolspin), resuspended in a small volume of filtered seawater and counted (Coulter counter, model ZB) prior to feeding to rotifer cultures.

Culture of rotifers

Stock cultures of Brachionus plicatilis (GS 74 strain), maintained at School of Ocean Sciences, were used to innoculate batch cultures of rotifers. Rotifers were cultured in 0.2μm-filtered, U.V. irradiated seawater at salinities of 25 - 32 ^{o}/oo and ambient

laboratory temperature (19 - 23 ^{o}C). Newly initiated rotifer cultures were fed daily with Rhinomonas reticulata, produced in semi-continuous batch culture and concentrated by centrifugation, as described above. For the first enrichment experiment only, a feeding regime of 200 - 400 cells μl^{-1} day^{-1} was maintained until immediately prior to the experiment. For subsequent experiments, cultures were started using a feeding regime of 200 cell μl^{-1} day^{-1} of R. reticulata supplemented with 0.5g bakers yeast million rotifers $^{-1}$ day^{-1}. Using this regime sustainable rotifer densities of 400 - 800 ml^{-1} were achieved in 25 l cultures in polythene carboys of the same type use for algal cultures. Two days prior to use in experiments rotifers from these cultures were harvested by siphoning onto an 80μm sieve, transferred to clean seawater at a density of 400ml^{-1}, and fed only bakers yeast at 0.5g million^{-1} day^{-1}.

Effect of dietary lipid content and feeding regimes on fatty acid composition and total lipid content of rotifers.

The effect of two spray-dried microencapsulated diets (supplied by Frippak Feeds, Findon, Aberdeen) containing total lipid at 18.2% of dry weight (CAR) and 33.6% of dry weight (Booster) on lipid composition of rotifers was investigated at three feeding rates and over four sampling times. Prior to the experiment, algal-fed rotifers from the stock culture were harvested and resuspended in clean seawater (25 ^{o}/oo, 20^{o}C). After 8h rotifers were collected by siphoning, taking care to avoid faecal material. 200ml of cleaned rotifer suspension (density = 360 ml^{-1}) was transferred to each of 14 round bottom flasks, 250 ml volume, with gentle aeration supplied through a glass pipette. Experimental treatments were fed one of the two enrichments diets at rates equivalent to 0.5g, 1.0g and 2.0g per million rotifers. Two flasks were allocated to each treatment and feeding level, with two unfed control flasks.

At intervals of 1h, 4h, 8h and 12h from the start of feeding 20ml samples were removed from each flask, washed over a 65μm mesh with 1 l of filtered seawater to remove excess diet and then with 3.9% ammonium formate solution to remove salt. Sample were examined under binocular microscope to ensure that all diet had been removed and then immediately frozen at -20^{o}C. All samples were freeze-dried prior to analysis.

Initially it had been intended to use one replicate sample for total lipid analysis and one for fatty acid composition analysis. However, the amount of material collected was found to be inadequate for total lipid analysis and the experiment was repeated in part. Rotifers were collected and stocked in 200ml flasks as described above. In this case, only the 1g million rotifers^{-1} feeding level was used and the entire rotifer population of each flask was harvested after 4h feeding, with an unfed control. 100ml samples were removed from each flask, thoroughly washed with

distilled water, transferred to glass vials and frozen at -20 ^{o}C.

Following the results of the previous experiment a third experiment examined the effect of the high-lipid diet (Booster) on the lipid content of rotifers during an extended enrichment period. After being fed only yeast for 48h prior to the experiment, rotifers were harvested by siphoning onto an 80μm sieved, washed with filtered seawater, and resuspended in seawater (32 ^{o}/oo). The volume of the culture was adjusted to give a density of 400 rotifers ml^{-1}. 2 l of the rotifer suspension was used to stock each of four round-bottom flasks, capacity approximately 2.2 l, with aeration supplied by glass pippetes. 1 g of the Frippak Booster diet was hydrated in filtered seawater and stirred for 30 min. Two flasks were fed the Frippak diet at 0.5g million rotifers^{-1}, calculated volumetrically, and two flasks remained unfed. At 0h, 4h, 8h and 16h after addition of diet to the cultures, 100ml samples were removed from each flask and washed and frozen as described for the previous experiment.

Effect of enrichment with Frippak Booster on rotifer population density.

Observation of rotifer cultures in the previous experiments indicated that there might be a drop in rotifer density during enrichment feeding. An experiment was carried out to test if this was the case. Rotifers cultures were grown using Rhinomonas reticulata and bakers yeast as described previously. Rotifers were harvested by siphoning over a 65μm sieve, washed and resuspended at a density of 600ml^{-1} in 2 l of U.V. sterilised, filtered seawater. Six 250 ml capacity round flasks were stocked with 200ml of rotifer suspension and aeration supplied through glass pipettes. Frippak Booster was hydrated and added to three flasks, with three flasks unfed. Five 50μl samples were taken from all flasks before addition of diet (0h) and at 4h, 8, 13h, 16h and 25h afterwards. Samples were examined under a binocular microscope and numbers of live rotifers recorded.

Fatty acid composition analysis

Lipids were extracted from dried and fresh samples in 10ml chloroform/ methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) (Folch et al. 1957). After 20 min at 4^{o}C, samples were filtered through a pre-washed No.4 paper. 0.2 volumes of 0.017% aqueous magnesium chloride was added and samples flushed with oxygen-free nitrogen (OFN). Samples were then centrifuged at 2000 r.p.m. (800g, MSE Centaur 1 centrifuge) and the upper phase discarded and twice replaced with Folch upper phase (chloroform: methanol:water, 3:48:47 v/v). The lower phase was then evaporated (Büchi Rotorevaporator) at 30^{o}C and the lipid redissolved in approximately 1ml of chloroform.

To methylate fatty acids, extracted lipids were heated to 100^{o}C in 1ml chloroform with 2 ml of 14% boron triflouride for 1 h. After cooling, the vial

contents were shaken with 3 ml water and 6 ml pentane. After settling the upper pentane layer was removed, replaced with fresh pentane and the process repeated. All pentane washes were collected and evaporated in OFN and fatty acid methyl ester residue dissolved in hexane.

Fatty acid methyl esters were separated on an Alltech Cebowax capillary column in a Carlo Erba Vega 6180 gas chromatograph equipped with a flame ionisation detector. Output was recorded on a Hewlett Packard 3390A integrator. Peaks for individual fatty acid methyl esters were identified by comparison with a cod liver oil standard. A 1μg calibration standard of 23.0 C fatty acid was injected into the column with each sample. However, as there was some doubt as to the efficiency of the freeze-drier in removing ammonium formate, samples were not weighed prior to analysis. Hence it was impossible to use the 23.0 standard to calculate the fatty acid content of the samples by weight. It was possible, however, to make some estimate the weight of each fatty acid per rotifer, based on the number of rotifers in each sample. Given the potential variability in losses during enrichment, harvesting, washing and lipid preparation it was decided not to use such estimates as the basis for comparison of treatments. Hence fatty acids were quantified as percentages of total area of identified fatty acids for each sample.

Total lipid analysis

Total lipid contents of freeze-dried rotifer and diet samples were determined using a modification of the Boehringer Mannheim total lipid test kit procedure (Boehringer Mannhiem, Uckfield, E. Sussex). Samples of 2 - 6 mg were weighed using a microbalance (Cahn C-31). Samples were dissolved in 2.0 ml of concentrated sulphuric acid at 100^{o}C. A standard was prepared using the same procedure, using 50μl of a solution of 10mg lipid.ml^{-1} in ethanol. After cooling, triplicate sub-samples of 50μl of the reaction mixture were added to 2.5ml of colour reagent (phosphoric acid/vanillin). A blank was prepared with colour reagent and 50μl sulphuric acid. After 30 min, absorbances of samples and standards were read against the blank at 530 nm.

Growth of carp larvae on enriched and unenriched rotifers

Eggs of mirror carp, Cyprinus carpio, were supplied by Munton and Fison PLC, Stowmarket, Suffolk. After hormonally induced spawning, eggs attached to nylon matting were transported to Menai Bridge in approximately 5 l of water in polythene bags. On arrival, matting and any loose eggs were transferred to a 10 l aquarium. A continuous supply of water was trickled into the aquarium from a recirculation system maintained at 24^{o}C, with outflow returning to the system through a 250 μm mesh. First hatching was observed after 72h and all viable eggs hatched within the

following 24h. Once mouth development and swim-bladder inflation had been observed, salinity was slowly increased to 5 ^{o}/oo to provide a more favourable medium for rotifers. At the start of the experiment, 25 larvae were transferred to each of nine 2l round flasks maintained at 25^{o}C in a water bath. Three flasks were allocated to each dietary treatment.

Rotifers were grown in 25 l polythene carboys, at 32 ^{o}/oo salinity, using Rhinomonas reticulata supplemented with bakers yeast until two days prior to the start of each experiment. Thereafter only bakers yeast, fed at 0.5g.million rotifers^{-1}, was used to maintain cultures. Each evening rotifers for the following day were harvested and resuspended in 15 ^{o}/oo seawater. Rotifers were harvested from these low salinity stock cultures four times daily, washed with filtered seawater and resuspended in 15 ^{o}/oo seawater. After each harvest, rotifers were enriched for 4h with the appropriate diets at 0.5g.million rotifers^{-1}, and then washed prior to feeding to larvae.
Enrichment regimes were;

1) Unenriched rotifers
2) 4h with Frippak CAR
3) 4h with Frippak Booster

Larvae were maintained on rotifers from the three enrichment regimes for 8 days, with total water replacement one each day. In all cases the feeding rates per flask were;

Days 1 - 3 ; 3 feeds of 20,000 rotifers
Days 4 - 6 ; 4 feeds of 20,000 rotifers
Days 7 - 8 ; 4 feeds of 30,000 rotifers

On day 9 all fish were killed with excess MS-222 and fork lengths and wet weight recorded. Five fish from each treatment were used for fatty acid composition analysis. After gut contents were carefully removed under a binocular microscope, to avoid contamination of samples with food material, carcasses were placed in cold chloroform:methanol containing BHT and stored at -20^{o}C until analysed.

RESULTS

Fatty acid composition and total lipid content of enriched and unenriched rotifers

Fatty acid composition and total lipid content of Frippak CAR and Booster diets are presented in Table 1. The two enrichment diets had similar fatty acid compositions, with high levels of 20.5n-3 and 22.6n-3 present in both (8.38% and 11.08% in Booster, 11.95% and 8.03% in CAR). However, the total lipid content of the Booster was higher (28.5%) than that of the CAR (18.2%).

Results of fatty acid analyses of rotifers from the three dietary treatments are presented in Tables 2a-c. Preliminary statistical analysis on data for 20.5n-3, 22.6n-3 and total n-3 HUFA contents of rotifers consisted of two-way ANOVA between treatments and across sampling times and, for the enriched treatments only, two-way ANOVA between treatments and across feeding levels. ANOVA tables are presented in Appendix 2, and show that for the first analysis there were significant differences between treatments in 20.5n-3 content ($p<0.001$), 22.6n-3 content ($P<0.001$) and n-3 HUFA content ($p<0.001$) but no significant effect of sampling time. In the second analysis there were again significant differences between treatments for 20.5n-3 ($p = 0.027$), 22.6n-3 ($p<0.001$) and n-3 HUFA ($p = 0.001$), with no significant effect of feeding levels and no significant interaction terms. Consequently, data from all feeding levels and sampling times were pooled prior to one way ANOVA between treatments. ANOVA tables are shown in Appendix 2, and indicate significant differences ($p<0.001$) in levels of 20.5n-3, 22.6n-3 and n-3 HUFA between the dietary treatments. Figure 1a shows mean values for pooled data from each treatment. Scheffé's method of pairwise comparison of means indicates that both enrichment diets were equally effective in significantly raising the percentage levels of 20.5n-3 in rotifers compared to the control treatment ($p<0.05$). In contrast only the Booster diet produced a significant increase in the percentage level of 22.6n-3 ($p<0.05$). Both enrichment diets significantly increased the percentage level of total n-3 HUFA compared to the control, but with the Booster diet being significantly more effective than CAR ($p<0.05$).

The total lipid contents of rotifers after 4h enrichment are shown also in Figure 1a. Lipid content of rotifers fed Booster diet for 4h was found to be significantly higher (20.9%) that of unfed rotifers (16.6%, $p<0.05$) while total lipid content of rotifers fed CAR for 4h (15.03%) was not significantly different from that of the unfed control. Plate 1 shows examples of rotifers from each of the dietary treatments, with an accumulation of lipid droplets around the gut apparent in animals fed the Booster diet, but not in those from the other treatments. Figure 1b shows changes in total lipid content of rotifers during 18h feeding on the Booster diet. As in the previous experiment there was a significant increase in total lipid content after 4h from the start of feeding. Thereafter total lipid content dropped significantly, but remained significantly higher than in rotifers prior to enrichment.

Effect of enrichment on population density

Figure 2 shows changes in population density of rotifers from cultures fed Frippak Booster and unfed control cultures. Analysis of co-variance on rotifer counts in treatments and replicates with time showed that there were no significant interaction

between treatment and replicate terms (Appendix 2). Hence data from the three replicates for each treatment were pooled prior to linear regression analysis. Suitability of the data for fitting to linear models was tested using the runs and sign test facilities in the Fig-P graphics and curve-fitting software package (Biosoft, Cambridge). Linear regression was carried out using the Minitab (version 6) statistics software package. No significant relationship was found between rotifer density and time for the unfed control cultures ($F = 0.2$, $p = 0.657$). In contrast, there was a significant ($F = 22.9$, $p < 0.001$) decline in rotifer density during the enrichment period (slope = -0.51 rotifers h^{-1}, $t = -4.79$, $p < 0.001$). Although this represents a low rate of decline, it would predict losses of 29% of original rotifer numbers after 16h enrichment. Examination of cultures during enrichment revealed dead or inactive rotifers not observed in the unfed control.

Effect of rotifer enrichment on growth of carp larvae.

Between 2 to 5 larvae in each flask did not start feeding and died within 2 days of the start of the experiment. As mortalities were evenly distributed between treatments, these mortalities were not replaced and all remaining larvae survived throughout the experiment. Nested ANOVA of length and weight data between treatments and replicates within treatments indicated no significant variation between replicates (Appendix 2). Hence, data from the three replicate flasks for each treatment were pooled prior to oneway ANOVA between treatments, followed by Tukey's pairwise comparison of means. Figure 3 shows mean lengths and weights of pooled samples of 30 carp larvae after nine days feeding on each of the three dietary treatments. There was no significant difference in length between larvae from the two enrichment treatments, which both produced larvae which were significantly longer than those from the control treatment ($p < 0.05$). However the larvae fed rotifers enriched with Frippak booster were significantly heavier than those fed rotifer enriched with CAR ($p < 0.05$), with both groups being significantly heavier than the control ($p < 0.05$).

Table 3 shows the fatty acid composition of rotifers used in the three feeding regimes and of carp larvae fed them. The fatty acid composition of control rotifers was dominated by high percentages of 16.7n-7 (26.03%), 18.1n-9 (24.97%) and 18.1n-7 (11.19%). Rotifers enriched with both diets contained higher percentage levels of 16.0, 18.0, 18.2n-6, 20.5n-3 and 22.6n-3 fatty acids than the control. Total n-3 HUFA was highest in rotfers fed Frippak Booster (24.78%) compared to 15.81% in rotifers fed CAR and 6.93% in the control. Rotifers fed the Booster diet were higher in total lipid content (20.7%) than those fed CAR (15.3%) or the control (14.6%).

The fatty acid composition of carp larvae reared on the control rotifers or

rotifers fed CAR were dominated by high levels of 16.0, 16.7n-7 and 18.1n-7, while larvae reared on rotifers fed the Booster diet contained a lower percentage of 16.1n-7. Larvae reared on rotifers fed the Booster diet contained very high levels of 22.6n-3 (15.5%) compared to 3.67% in the control and 4.53% in larvae reared using rotifers fed CAR. Only low levels of 20.5n-3 were recorded in larvae from all three treatments, despite the high dietary levels of the fatty acid in the enriched rotifers. The total lipid contents of carp larvae from the three treatments did not vary significantly, and ranged from 8.2% to 9.3% of dry weight.

DISCUSSION

In the first experiment, the percentage contents of n-3 HUFA, particularly 22.6n-3 in rotifers fed Rhinomonas reticulata are fairly high for algal-fed rotifers even after 9 h starvation. In comparison, rotifers fed marine Chlorella can contain very high levels of 20.5n-3 (up to 27%), but typically are very low in 22.6n-3 content (Watanabe et al. 1983a). Similarly Nannochloropsis spp. have been found to produce high levels of 20.5n-3 (6.5%) but low 22.6n-3 (0.8%) in rotifers (Ben-Amotz et al. 1987. Mourente et al. 1993). Chlorella stigmatophora and Chaetoceros gracilis have been found to produce relatively low levels of both 20.5n-3 and 22.6n-3 (Ben-Amotz et al. 1987). Whyte and Nagata (1990) found that Isochrysis galbana produced high levels of 22.6n-3 (8.53%) in rotifers, but little 20.5n-3 (2.2%). Of the range of algal species tested by Whyte and Nagata (1990) only Thalassiosira pseudonana produced high levels of 20.5n-3 (20.5%) and 22.6n-3 (6.02%) in rotifers.

The more effective performance of the Frippak Booster in raising n-3 HUFA, and particularly 22.6n-3 content of rotifers is attributable to its higher total lipid content compared to CAR, given the similar fatty acid composition of the diets and the fact that the same ingredients had been used in their formulation (Frippak Feeds, pers. comm.). The accompanying increase in total lipid content of rotifers fed Frippak Booster for 4h is similar to that observed in rotifers fed the commercial lipid-emulsion diet Super Selco* (diet lipid content 93%), and higher than that produced by enrichment with the microparticulate diets Protein Selco* (diet lipid content 30%) or Dry Selco* (diet lipid content 56%) (Fernández-Reirez et al., 1993). Higher lipid content (up to 27%) has been reported in rotifers after primary and secondary enrichment with Super Selco (Kjφrsvik et al., 1991).

Changes in n-3 HUFA content of rotifers fed Frippak CAR in the present study were similar to those recorded by Budd (1989) using a spray-dried CAR diet and by Walford and Lam (1987) using a protein cross-linked microencapsulated diet of

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* The Selco range of diets are products of Artemia Systems, Belgium

similar lipid composition. Changes in percentage content of n-3 HUFA in rotifers fed the Frippak Booster diet were similar to those reported by Imada et al. (1979) using yeast which contained 12-16% total lipid and 34-36% n-3 HUFA, and by Izquierdo et al. (1989) using a cod liver oil emulsion. Fernández-Reirez et al. (1993) found that n-3 HUFA content of rotifers, expressed as percentage of dry weight, increased with lipid content of enrichment diet. Thus differences in relative fatty acid contents between treatments in the present study probably indicate even greater differences in absolute n-3 HUFA levels, particularly given the differences in lipid content of rotifers from the two enriched treatments.

In the present study, enrichment feeding level and duration did not affect relative content of n-3 HUFA in rotifers. Using feeding levels of 0.05 to 2.0g million rotifers^{-1}, Budd (1989) found that feeding Frippak CAR for 8h at 0.5g million rotifers^{-1} produced the highest levels of n-3 HUFA in rotifers, though differences were not statistically tested. As present results have shown a significant decline in rotifer numbers during enrichment, feeding at 0.5g million rotifers^{-1} for a short period (4h) would appear to be the optimal protocol for the usage of the Booster diet, at least within the range of treatments tested.

However, a lower effective feeding level, with less deleterious effect on rotifer population density, might usefully be determined. The CAR diet, on which the Booster diet is based, is designed as an algal replacement for penaeid shrimp larval culture and is typically used at feeding densities of 8 - 10 mg l^{-1} compared to the 200 mg l^{-1} required to feed at a rate of 0.5g million rotifers^{-1} in the present study. However, such high feeding rates are typical of rotifer culture and enrichment practices. Lubzens (1987) found that optimal population growth in rotifers fed Chlorella stigmatophora was achieved at cell densities of 2000-8000 cells μl^{-1}, compared to typical algal feeding rates for penaeid larvae of around 50 cells μl^{-1}. Other commercially available enrichment diets, such as the Selco range, have been used at 0.6g / million rotifers (= 0.38g l^{-1}) by Fernández-Reirez et al. (1993), and experimental diets at 0.5g million rotifers^{-1} by Rainuzzo et al. (1989). Bakers yeast has been used to culture rotifers at feeding rates of 0.14g -1.0g / million rotifers (Walford 1990, Fernández-Reirez et al. 1993, present study).

It may be that accumulation of leached dietary components at the high feeding rates used leads to the drop in population density. However, even when used at lower feeding rates, nylon-protein cross-linked and spray-dried microencapsulated diets have failed to support population growth of rotifers (Teshima et al. 1982, Budd 1989). Walford (1990) attempted primary enrichment using protein cross-linked microcapsules as a supplement to yeast, but attributed collapse of rotifer cultures to pollution from the artificial diet. The high lipid content of the Booster diet is unlikely

to have contributed to the drop in rotifer density observed during enrichment as Budd (1989) reported similar losses during enrichment with Frippak CAR at 0.5g-2.0g million rotifers^{-1} over 16h, though no losses resulted from feeding the same diet at 0.05g million rotifers^{-1}.

The yeast-grown rotifers fed to the control treatment of carp larvae contained low percentages of 18.3n-3, 18.2n-6, 20.5n-3 and 22.6n-3 fatty acids. Typically yeast-grown rotifers lack 18.3n-3 (Lubzens et al. 1987), and the presence of this polyunsaturated fatty acid may result from the initial use Rhinomonas reticulata in culturing the rotifers, as indicated by the fatty acid composition of rotifers fed the alga exclusively in the first experiment of the present study. The presence of low levels of n-3 HUFA is to be expected in yeast-grown rotifers (Lubzens 1985), though levels of 20.5n-3 and 22.6n-3 in yeast-fed rotifers in the present study are approximately double those reported by Walford (1990) for rotifers grown exclusively on bakers' yeast for 30 days. Watanabe et al. (1983a) reported levels of 1.9% 20.5n-3 and 0.2% 22.6n-3 content in yeast-fed rotifers. In contrast Denrinos and Thorpe (1987) found higher levels of 22.6n-3 (2.2%) and only trace amounts of 20.5n-3 in yeast-fed rotifers.

As in the present study, the n-3 HUFA content of larvae of the freshwater species Gobio gobio and Perca fluviatilis, have been found to reflect the n-3 HUFA content of Brachionus calyciflorus (Awaïss 1991). Similarly, the n-3 HUFA content of larvae of the marine bream species Pagrus major and Sparus aurata have been found to increase with the n-3 HUFA content of enriched rotifers (Izquierdo et al. 1989, Mourente et al. 1993). The accumulation of 22.6n-3 in carp larvae fed yeast-reared rotifers in the present study indicates the elongation and desaturation of dietary 18.3n-3 and 20.5n-3, as would be expected from the results of Csengeri et al. (1987), Farkas et al. (1977) and Neto et al. (1993). The presence of some n-3 polyunsaturated fatty acids in the diet of the control group of fish explains the absence of n-9 HUFA in the fish, which might be expected to accumulate in carp larvae fed totally n-3 HUFA deficient diets (Neto et al. 1993).

In the present study, enrichment with Frippak CAR did not increase the total lipid content of rotifers, but significantly increased n-3 HUFA content. The significant improvement in length and weight observed in carp larvae fed the CAR-enriched rotifers compared to the control suggests that the fatty acid content of the yeast-fed rotifers was limiting to growth - which was improved by the supplementation of n-3 HUFA in the CAR enriched rotifers. Similar improvements in growth in response to increasing dietary 20.5n-3 and 22.6n-3 have been reported in Oncorhynchus mykiss (Takeuchi and Watanabe 1976, 1977) and Coregonus lavaretus (Watanabe et al. 1989). Neto et al. (1993) have estimated that first-feeding

carp larvae can survive and grow to 100mg weight when fed diets containing as little as 0.05% (of dry weight) n-3 fatty acids, with no improvement in growth on addition of methyl linolenate (methyl 18.3n-3) at 0.25% or 0.5%. However, in the same study a diet with n-3 fatty acid content of 0.3% of dry weight (produced by addition of 1% cod liver oil which is high in 20.5n-3 and 22.6n-3) supported better growth.

In the present study, carp larvae fed rotifers enriched with Frippak Booster, which were high in lipid content, gained significantly more weight than those fed CAR-enriched rotifers. The accumulation of 22.6n-3 and extra weight gain in larvae fed the Booster-enriched rotifers indicates the effective transfer to the larvae of at least some of the lipid accumulated by rotifers fed the Booster diet.

As the total lipid content of larvae in the present study did not differ significantly between treatments, the differences in final weight of larvae between treatments is not attibutable to lipid storage. The feeding rates used in the present study were higher than the maximum used by Lubzens et al. (1987) in feeding koi carp larvae (2400 rotifers / larva / day), and were always to excess, so that food availability was not limiting in any of the treatments. It is possible, however, that the extra weight gain observed in larvae fed Booster-enriched rotifers might partly result from protein 'sparing' through supply of extra lipid - as secondary enrichment of rotifers with Frippak booster has little effect on their total protein content (Theodoru 1992), and the results of Fernández-Reirez et al. (1993) indicate that enriched rotifers accumulate both triaclyglycerols and phospholipids. Jauncey (1979) found that increasing dietary lipid content from 12% to 18% resulted in increased specific growth rate in mirror carp, and that dietary protein content could be reduced at the higher lipid level without imparing growth. It is impossible to quantify a protein 'sparing' effect in the present study, though it is clear from the improved growth larvae fed CAR-enriched rotifers that the improvement in length and at least part of the improvement in weight in carp larvae fed Booster-enriched rotifers is attributable to dietary fatty acid composition rather than total lipid content.

Most of the available information on fatty acid requirements of marine fish is derived from studies on juveniles as larvae of marine species do not generally feed well on formulated diets. Some recent studies have manipulated the fatty acid content of live feeds in order to determine minimum dietary requirements for n-3 HUFA. Such studies have indicated minimum contents of n-3 HUFA (in $\mu g\ mg^{-1}$) of live food which will support growth and suvival of Sparus aurata (5.1 $\mu g\ mg^{-1}$, Koven et al. 1990) and for Morone saxatilis (5.7 $\mu g\ mg^{-1}$, Tuncer and Harrel 1992).

In the present study fatty acids were quantified as percentage of total fatty acids, rather than in absolute amounts. However, using the C_{23} internal standard it is possible to make an estimate of n-3 content of 30.15 μg individual^{-1} for rotifers

enriched with Frippak Booster for 4h. Using a dry weight estimate of 0.4 μg for an enriched rotifer (Fernández-Reirez et al. 1993), this gives an estimated n-3 HUFa content for Booster-enriched rotifers of 14.9 μg mg^{-1} dry weight. Given the potential losses during sample processing it is likely that this estimate is low, but it still compares favourably with values for other commercial enrichers (Fernández-Reirez et al. 1993) and is well above the known minimum requirements of marine fish larvae. Mourente et al. (1993) have demonstrated that growth in Sparus aurata larvae is correlated with 22.6n-3 content of rotifers and have suggested that larvae of this species have a poor ability to elongate and desaturate 20.5n-3. The estimated 22.6n-3 content of Booster enriched rotifers in the present study was 5.7 μg mg^{-1} dry weight, which is higher than the maximum value tested by Mourente et al. (1990). Since the present study was completed, Frippak Booster diet has been shown to be highly effective in promoting growth in larvae of marine species, including Sparus aurata (Pousao-Ferreira and Soares, 1991).

In contrast to marine species, the potential benefits of the application of live food enrichment practices in the culture of freshwater fish has largely been overlooked. This may be in part due to the less stringent fatty acids requirements of freshwater larvae, and also because typically extensive larval culture methods are used for non-salmonid freshwater species. In intensive culture of carp larvae a range of live feeds are used, including Artemia nauplii, Daphnia spp. and Moina spp., in addition to formulated feeds (Bryant and Matty 1980, and references therein, Charlon and Bergot 1984, 1986, Michaels 1988). The use of rotifers as a supplement to dry feeds in the intensive rearing of freshwater cyprinid larvae has been advocated by Lubzens et al. (1984,1987), and present results demonstrate that enrichment diets can be beneficial in improving the growth rate of carp larvae reared using rotifers. Although not compared directly, the growth of larvae receiving Booster-enriched rotifers was superior to that recorded in mirror carp larvae fed Artemia nauplii under similar conditions within the same experimental system (Chapter 3, present studies).

Present results with rotifers indicate that that oil-rich spray-dried microencapsulated diets might be used to improve the nutritional quality of other live food organisms, and hence might be more widely applied in culture of freshwater fish species. As in the case of rotifers, both Moina sp. and Daphnia sp. can be very low in 20.5 n-3 and 22.6 n-3 content, and the n-3 HUFA content of Moina sp. can be greatly enhanced by short-term feeding on lipid emulsions (Watanabe et al. 1983). The fatty acid composition of Artemia nauplii can be also improved with enrichment diets (Table 6, Chapter 1), and pre-feeding Artemia nauplii with a microencapsulated diet has been shown to improve growth of carp larvae (Grove and Fletcher 1988).

Table 1. Fatty acid composition (% of total fatty acids) and total lipid content (% of dry weight, +/- s.d.) of Frippak CAR and Frippak Booster diets.

Fatty acid	Frippak Booster	Frippak CAR
14	4.19	5.01
15	0.37	0.26
16	16.72	17.58
16.1n-9	0.29	2.22
16.1n-7	4.57	5.02
17	0.39	1.31
18	6.51	9.24
18.1n-9	13.08	9.92
18.1n-7	2.94	1.55
18.2n-6	6.24	5.05
18.3n-3	1.23	0.73
18.4n-3	2.57	3.24
20	0.21	0.34
20.1n-9	6.67	5.13
20.2n-6	0.45	0.25
20.3n-6	0.27	0.13
20.4n-6	2.94	1.73
20.5n-3	8.38	11.95
22.1n-11	9.11	8.44
22.4n-6	0.30	0.42
22.5n-3	1.49	2.46
22.6n-3	11.08	8.03

Totals

Saturates	28.40	33.73
Monoenes	36.66	32.28
Polyenes	34.94	33.98
(n-6)HUFA	3.51	2.27
(n-3)HUFA	24.75	26.42

Total lipid (% dry wt)	28.50 +/- 2.02	18.23 +/- 2.22

Table 2a. Fatty acid composition (% of total fatty acids) of rotifers enriched with Frippak CAR (lipid content 18.2% of dry weight). Samples were taken at 1h, 4h, 8h and 12h from start of feeding at three levels (0.5g, 1.0g and 2.0g / million rotifers).

Fatty acid	0.5g / million				1g / million				2g / million			
	1h	4h	8h	12h	1h	4h	8h	12h	1h	4h	8h	12h
14	7.81	6.73	6.46	6.34	4.16	7.92	6.92	5.82	5.63	6.65	5.51	6.09
15	1.20	1.55	1.64	0.94	1.28	1.55	1.40	0.91	1.33	1.48	1.07	0.97
16	15.39	18.37	17.29	19.77	18.87	17.56	17.94	13.24	18.44	11.55	15.59	15.82
16.1n-9	1.71	2.01	1.97	1.37	2.41	3.01	2.78	1.37	1.70	1.81	1.39	1.59
16.1n-7	2.61	3.90	4.81	6.63	3.42	5.13	5.58	7.60	3.49	6.22	5.39	6.86
17	0.57	0.81	0.47	0.50	0.76	0.56	0.53	0.43	0.70	0.59	0.50	0.39
18	6.34	7.17	5.63	5.50	7.83	5.29	4.96	5.12	6.57	5.69	4.71	4.52
18.1n-9	4.99	7.57	6.73	6.85	6.97	7.35	7.49	6.99	5.05	4.16	6.31	6.33
18.1n-7	2.78	3.02	3.12	4.60	3.00	3.27	3.45	4.47	2.63	3.93	3.15	4.03
18.2n-6	7.90	9.80	12.96	15.75	9.22	14.09	15.58	15.03	9.57	12.31	16.29	19.56
18.3n-3	10.14	6.36	6.80	6.27	7.53	7.30	6.16	7.02	8.10	9.05	7.01	6.68
18.4n-3	7.73	4.87	4.78	3.92	5.51	5.32	4.12	4.64	6.12	6.68	4.31	4.17
20	0.94	0.46	n.d.	0.24	0.42	0.48	0.29	0.21	0.40	0.28	0.27	0.18
20.1n-9	3.55	1.06	n.d.	1.03	2.15	1.55	1.28	1.87	1.86	2.34	1.62	1.18
20.2n-6	0.95	2.43	5.87	0.90	1.28	0.72	0.71	0.94	0.79	0.95	0.77	0.89
20.3n-6	0.82	0.47	n.d.	0.53	0.49	0.56	0.50	0.62	0.61	0.79	0.52	0.55
20.4n-6	2.07	2.06	2.05	1.62	1.68	1.51	1.40	1.79	1.65	2.10	1.40	1.45
20.5n-3	7.45	7.20	8.91	8.42	7.46	8.45	8.49	11.02	7.73	10.58	8.86	9.62
22.1n-11	1.67	0.98	0.47	1.00	1.28	0.75	0.83	0.94	1.23	1.12	0.92	0.77
22.4n-6	5.34	5.45	1.67	0.97	8.50	1.26	2.02	0.65	8.57	1.14	6.29	0.41
22.5n-3	3.05	2.28	2.08	2.12	0.81	1.17	2.07	2.75	2.56	2.80	2.50	2.40
22.6n-3	5.00	5.45	6.29	4.73	4.97	5.20	5.50	6.57	5.28	7.78	5.62	5.54

Totals

Saturates	32.25	35.09	31.49	33.29	33.32	33.36	32.04	25.73	33.07	26.24	27.65	27.97
Monoenes	17.31	18.54	17.10	21.48	19.23	21.06	21.41	23.24	15.96	19.58	18.78	20.76
Polyenes	50.45	46.37	51.41	45.23	47.45	45.58	46.55	51.03	50.98	54.18	53.57	51.27
(n-6)HUFA	8.23	7.98	3.72	3.12	10.67	3.33	3.92	3.06	10.83	4.03	8.21	2.41
(n-3)HUFA	15.50	14.93	17.28	15.27	13.24	14.82	16.06	20.34	15.57	21.16	16.98	17.56

Table 2b. Fatty acid composition of rotifers enriched with Frippak Booster (lipid content 28.5% of dry weight).
Samples were taken at 1h, 4h, 8h and 12h from start of feeding at three levels (0.5g, 1.0g and 2.0g / million rotifers).

Fatty acid	0.5g / million				1g / million				2g / million			
	1h	4h	8h	12h	1h	4h	8h	12h	1h	4h	8h	12h
14	7.74	5.45	4.00	6.43	7.81	6.64	4.93	5.25	6.32	6.19	5.88	4.15
15	1.47	0.90	0.80	1.05	1.09	1.62	0.77	0.82	0.84	0.98	1.09	0.84
16	17.68	16.43	13.76	13.19	17.35	15.64	11.61	11.11	12.40	12.21	16.75	13.33
16.1n-9	3.21	1.52	1.50	1.14	1.68	2.06	1.05	1.50	1.33	2.37	1.86	1.29
16.1n-7	3.48	3.51	4.05	6.81	3.69	3.97	4.65	5.05	4.18	4.98	5.46	4.96
17	0.70	0.51	0.39	0.47	0.55	0.41	0.34	n.d.	0.47	0.62	0.53	0.45
18	6.79	7.08	4.85	5.09	6.53	6.52	4.18	4.48	5.79	5.90	6.22	5.37
18.1n-9	9.34	9.44	9.81	6.96	8.88	10.52	9.86	10.37	8.90	10.40	11.41	10.02
18.1n-7	2.91	3.74	3.09	4.21	3.29	3.34	3.00	3.37	3.20	3.35	3.77	3.35
18.2n-6	9.21	9.98	10.04	13.96	9.53	9.82	11.27	11.39	9.71	9.31	10.53	10.83
18.3n-3	6.78	6.20	6.33	6.93	7.02	5.37	6.22	5.33	7.71	6.46	5.20	4.83
18.4n-3	5.30	5.90	5.01	4.66	5.76	4.92	5.64	4.69	6.65	5.80	4.40	4.47
20	0.32	0.29	0.27	0.21	0.09	0.39	0.14	0.17	0.18	0.19	0.28	0.26
20.1n-9	1.69	1.30	1.45	1.66	1.92	1.38	0.85	0.99	1.10	1.49	1.10	1.08
20.2n-6	0.92	0.68	1.07	0.90	0.69	0.81	0.71	0.79	0.73	0.84	0.80	0.82
20.3n-6	0.60	0.66	0.72	0.64	0.55	0.56	0.70	0.63	0.62	0.59	0.59	0.80
20.4n-6	2.19	3.23	3.94	2.54	2.34	3.34	4.83	4.84	3.11	3.78	3.61	4.17
20.5n-3	8.12	9.11	10.00	10.50	8.20	9.16	12.16	11.71	10.83	10.75	8.76	10.19
22.1n-11	2.61	2.05	1.34	0.38	2.35	2.55	0.48	1.68	3.39	0.58	1.89	1.59
22.4n-6	0.66	0.48	3.75	1.40	1.28	1.18	1.68	0.70	0.38	1.17	0.54	2.61
22.5n-3	1.08	2.55	3.23	2.83	1.94	1.22	2.91	3.08	2.69	2.29	1.19	3.81
22.6n-3	7.20	8.99	10.60	8.05	7.48	8.58	12.02	12.05	9.47	9.75	8.14	10.78

Totals

Saturates	34.70	30.66	24.07	26.44	33.42	31.22	21.97	21.83	26.00	26.09	30.75	24.40
Monoenes	23.24	21.56	21.24	21.16	21.81	23.82	19.89	22.96	22.10	23.17	25.49	22.29
Polyenes	42.06	47.78	54.69	52.41	44.79	44.96	58.14	55.21	51.90	50.74	43.76	53.31
(n-6)HUFA	3.45	4.37	8.41	4.58	4.17	5.08	7.21	6.17	4.11	5.54	4.74	7.58
(n-3)HUFA	16.40	20.65	23.83	21.38	17.62	18.96	27.09	26.84	22.99	22.79	18.09	24.78

Table 2c. Fatty acid composition (% of total fatty acids) of rotifers after 1h, 4h, 8h and 12h in the unfed control treatment.

Fatty acid	1h	4h	8h	12h
14	8.11	6.59	9.63	8.00
15	1.78	1.49	3.32	2.39
16	21.60	17.30	20.05	20.48
16.1n-9	2.68	3.71	4.62	2.94
16.1n-7	3.85	2.49	2.85	2.43
17	0.79	0.68	0.95	0.85
18	7.46	7.63	8.35	8.17
18.1n-9	6.52	7.21	7.49	6.90
18.1n-7	3.85	2.69	3.41	3.44
18.2n-6	9.84	7.45	8.59	8.97
18.3n-3	7.94	7.56	6.29	6.28
18.4n-3	5.94	5.28	2.99	3.05
20	0.49	0.39	0.26	0.60
20.1n-9	1.74	2.65	3.09	3.62
20.2n-6	0.92	0.84	1.30	0.78
20.3n-6	n.d.	1.03	0.63	0.62
20.4n-6	1.45	2.19	1.58	1.88
20.5n-3	7.16	6.18	4.13	4.13
22.1n-11	n.d.	3.78	1.35	1.33
22.4n-6	0.59	3.98	4.59	7.57
22.5n-3	1.89	2.89	1.65	2.43
22.6n-3	4.75	5.99	2.88	3.14

Totals

Saturates	40.23	34.08	42.56	40.49
Monoenes	18.64	22.53	22.81	20.66
Polyenes	41.13	43.39	34.63	38.85
(n-6)HUFA	2.69	7.20	6.80	10.07
(n-3)HUFA	13.80	15.06	8.66	9.70

Table 3. Fatty acid compositions (% of total fatty acids) of rotifers and 9 day old carp larvae. Rotifers were cultured using algae and yeast (control) and secondarily enriched for 4h with diet Frippak CAR and Frippak Booster. Carp larvae were reared from first feeding using rotifers from each treatment.

	Rotifers			Carp		
Fatty acid	Control	Frippak CAR	Frippak Booster	Control	Frippak CAR	Frippak Booster
14	1.92	2.91	6.40	0.23	0.41	0.41
15	0.43	0.61	0.66	0.10	0.08	0.18
16	6.21	16.04	16.38	20.41	17.07	22.01
16.1n-9	0.68	1.19	0.54	0.40	0.30	0.28
16.1n-7	26.03	12.09	11.01	15.33	10.33	5.76
17	1.28	1.93	1.08	0.16	0.77	0.30
18	3.39	7.97	9.24	11.23	10.00	9.62
18.1n-9	24.97	19.99	12.80	34.57	26.91	33.89
18.1n-7	11.19	4.65	5.27	6.64	4.14	2.20
18.2n-6	2.51	7.19	5.19	0.42	1.65	1.25
18.3n-3	1.87	2.59	1.19	0.17	0.82	0.39
18.4n-3	0.18	1.10	0.10	0.22	0.39	0.18
20	0.44	0.80	0.04	0.95	1.24	0.27
20.1n-9	3.79	4.27	2.57	1.46	11.65	1.44
20.2n-6	1.24	0.40	0.28	0.34	3.07	n.d.
20.3n-6	2.18	0.60	0.04	0.23	1.83	n.d.
20.4n-6	5.58	2.51	2.11	1.46	1.81	1.16
20.5n-3	2.23	6.80	12.08	0.98	0.73	2.05
22.1n-11	1.23	1.05	1.22	0.44	2.24	n.d.
22.4n-6	n.d.	n.d.	0.38	0.16	n.d.	n.d.
22.5n-3	1.32	1.03	2.04	0.45	0.46	1.08
22.6n-3	1.33	4.29	9.38	3.67	4.07	15.50

Totals

Saturates	13.67	30.26	33.79	33.07	29.57	32.79
Monoenes	67.89	43.25	33.41	58.83	55.59	43.56
Polyenes	18.43	26.50	32.79	8.10	14.84	21.62
(n-6)HUFA	7.76	3.11	2.54	1.86	3.64	1.16
(n-3)HUFA	6.93	15.81	24.78	5.49	6.48	19.21

Total lipid (%, +/- s.d.)	14.3 +/- 1.2	15.3 +/- 0.8	20.7 +/- 1.4	8.17 +/- 1.07	9.36 +/- 0.82	9.04 +/- 0.57

Figure 1a. n-3 HUFA (% of fatty acids) and total lipid (% of dry weight) content of unfed rotifers, and of rotifers fed Frippak CAR or Frippak Booster. HUFA data are pooled from three feeding levels and four sampling times (shown in Table 1), total lipid data are for samples taken after 4h enrichment. Values are means, error bars are standard deviations (n = 4-12 for each value). For each lipid component, treatments marked with different superscripts are significantly different ($p<0.05$, ANOVA followed by Scheffé's pairwise comparison).

Figure 1b. Total lipid content (% of dry weight) of rotifers fed Frippak Booster (0.5g / million rotifers) over 16h. Values are means of triplicate assays of two samples from each of two replicate cultures (n = 12 for each value). Values are means, error bars are standard deviations. Values marked with different superscripts are significantly different ($p<0.05$, ANOVA followed by Scheffé's pairwise comparison).

Figure 1a.

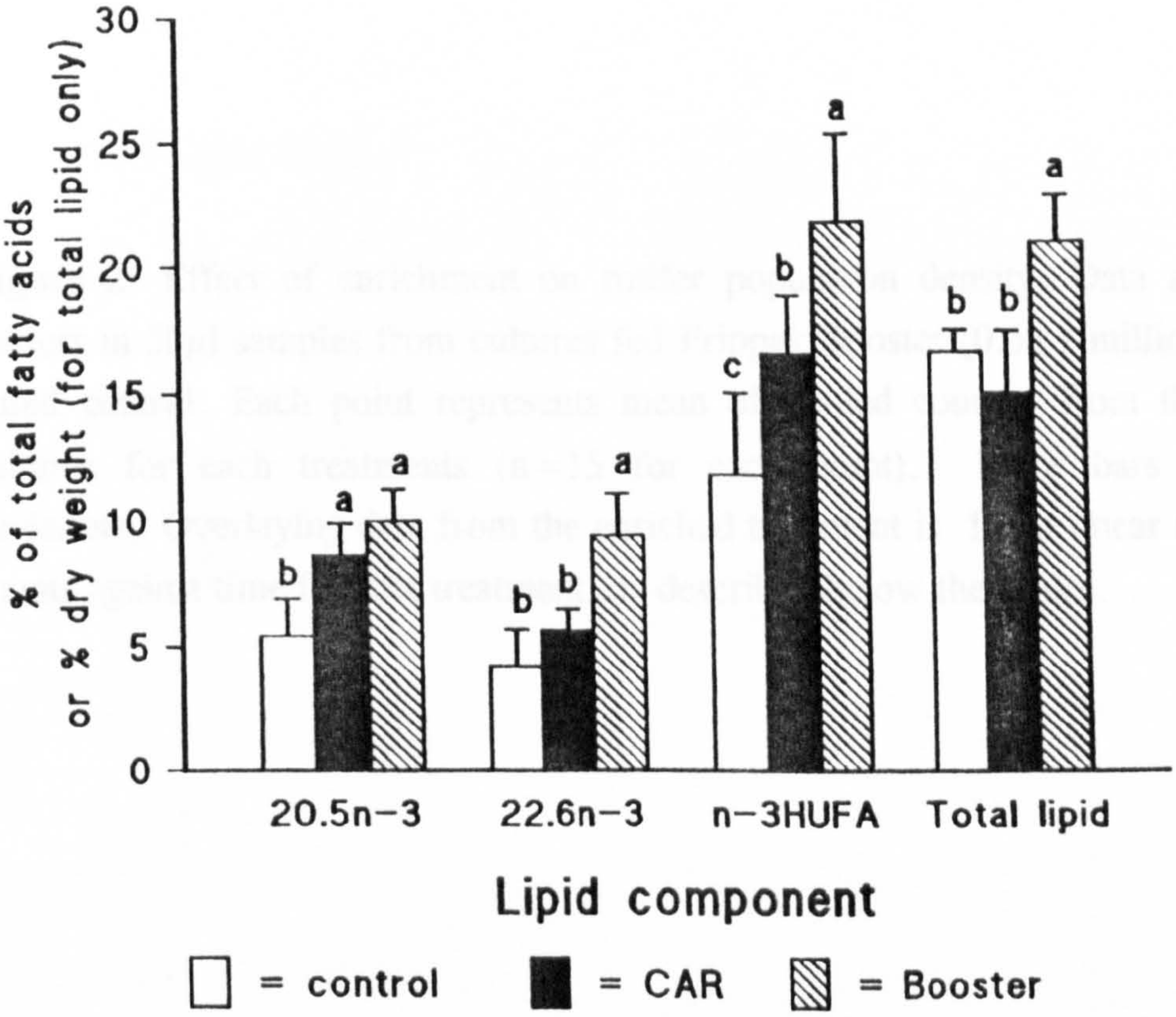


Figure 1b.

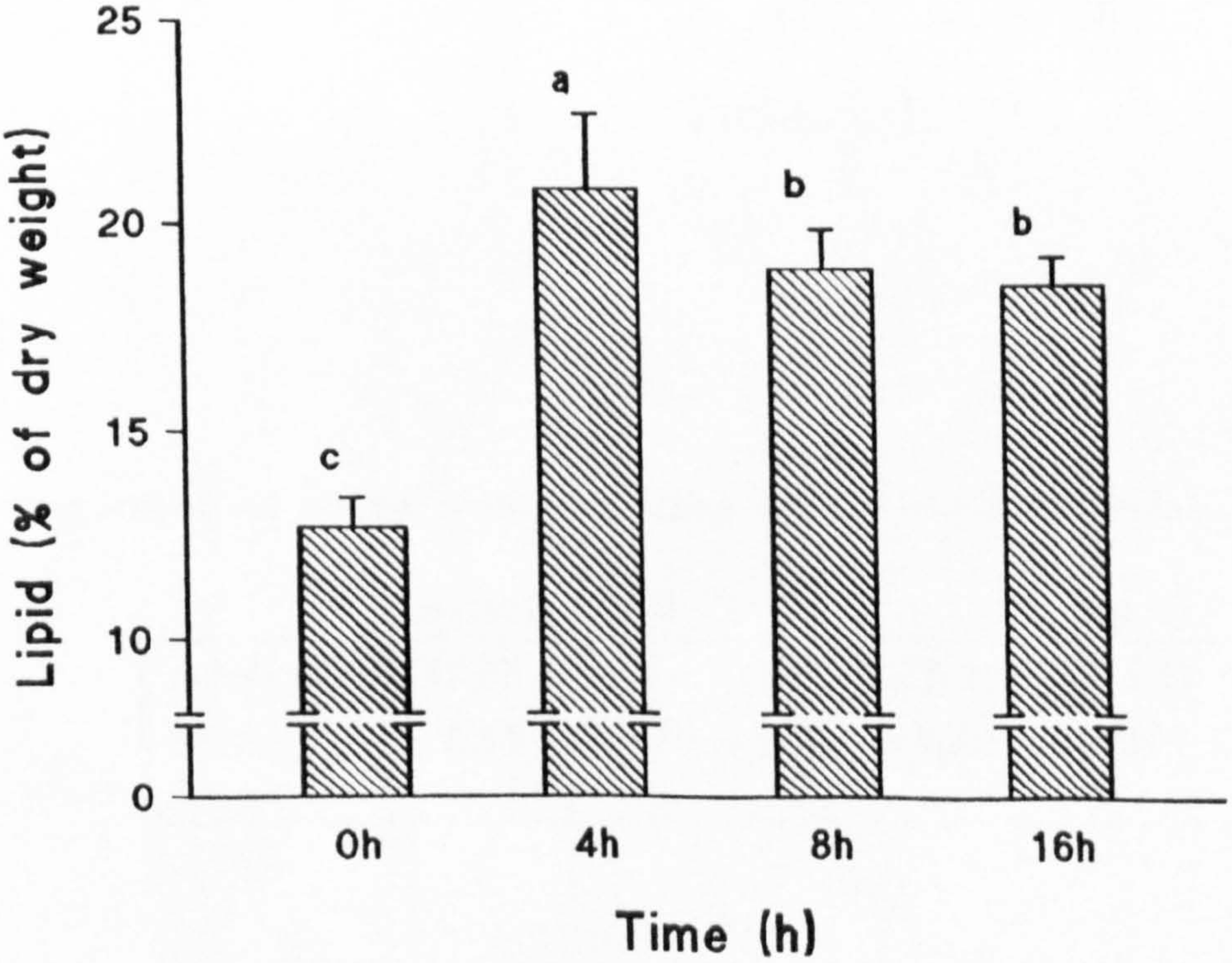
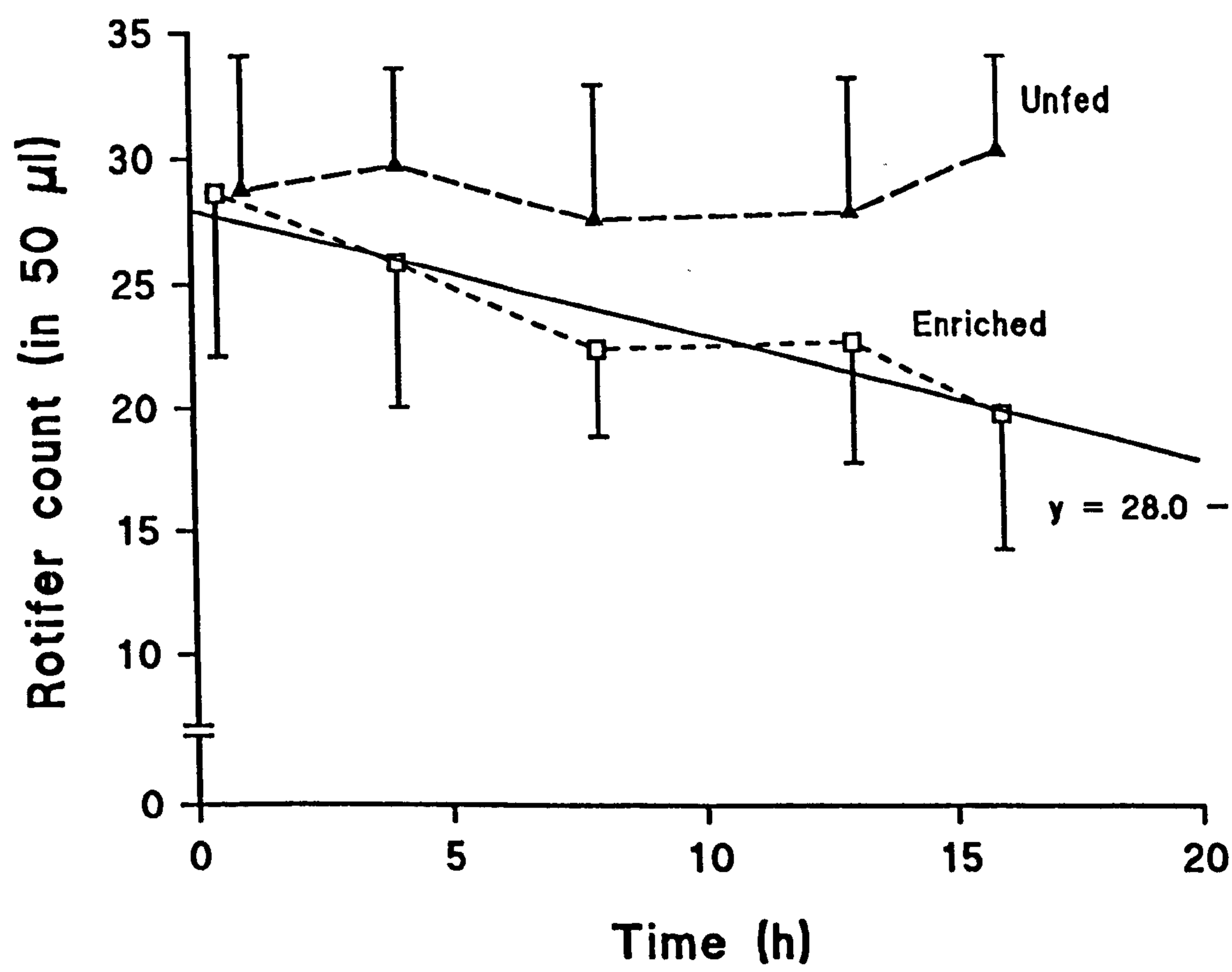


Figure 2. Effect of enrichment on rotifer population density. Data are counts of rotifers in 50μl samples from cultures fed Frippak Booster (0.5g / million rotifers) or unfed control. Each point represents mean of pooled counts from three replicate cultures for each treatments (n=15 for each point). Error bars are standard deviations. Overlaying data from the enriched treatment is fitted linear regression for counts against time for that treatment, as described below the figure.

Figure 2.



Regression of rotifer counts vs time for enriched treatment:

	Constant	95% CI	t	p
Intercept =	27.97	(+/− 2.12)	26.31	<0.001
Slope =	−0.51	(+/− 0.21)	−4.79	<0.001

Figure 3. Mean lengths (mm) and weights (mg) of 9 day old carp larvae reared using unenriched rotifers (control) or rotifers enriched with Frippak CAR or with Frippak Booster. Values are pooled from samples of 10 fish from each of three replicate flasks for each treatment ($n = 30$), error bars are standard deviations. Treatments marked with different superscripts are significantly different ($p<0.05$, ANOVA followed by Tukey's pairwise comparison).

Figure 3.

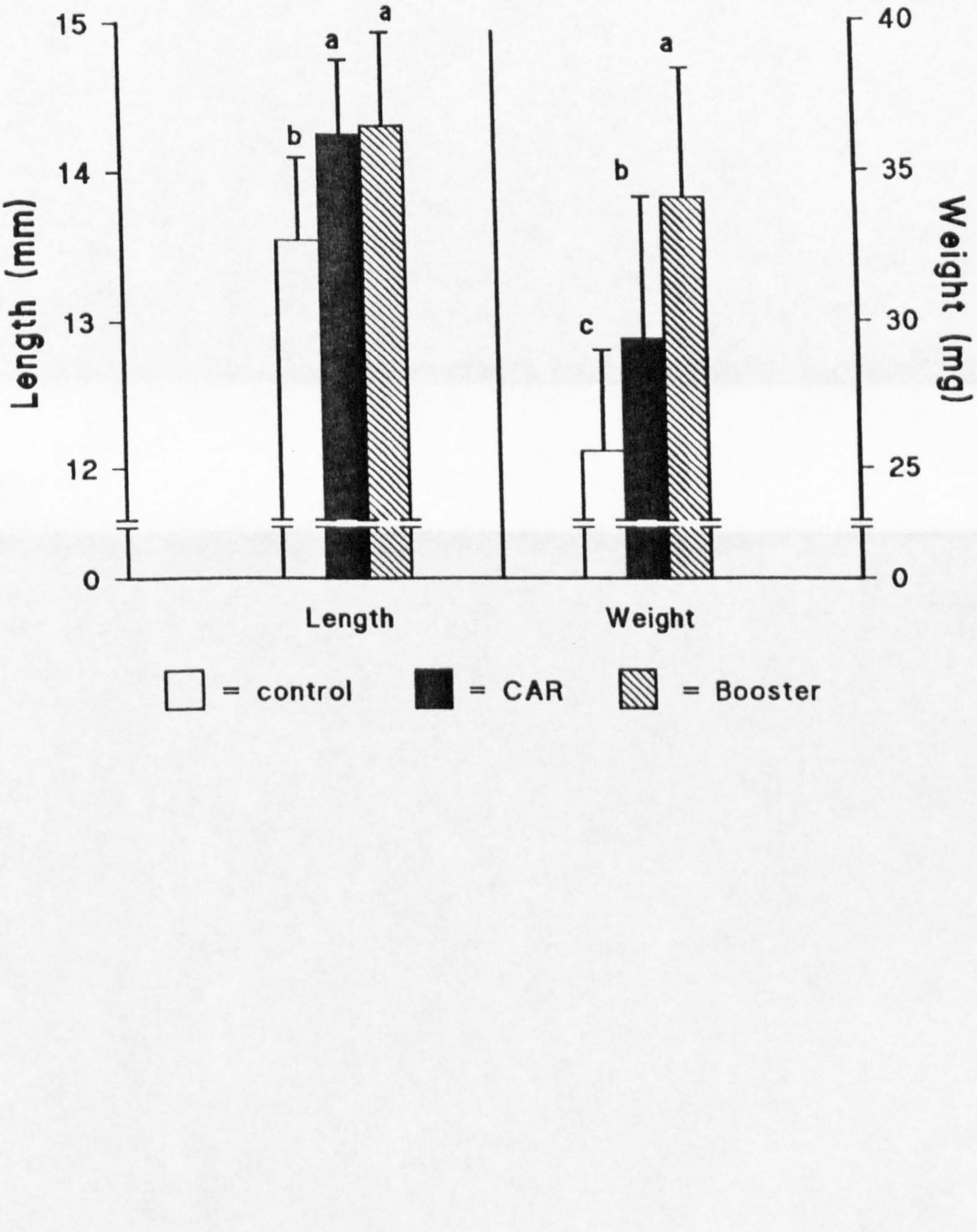
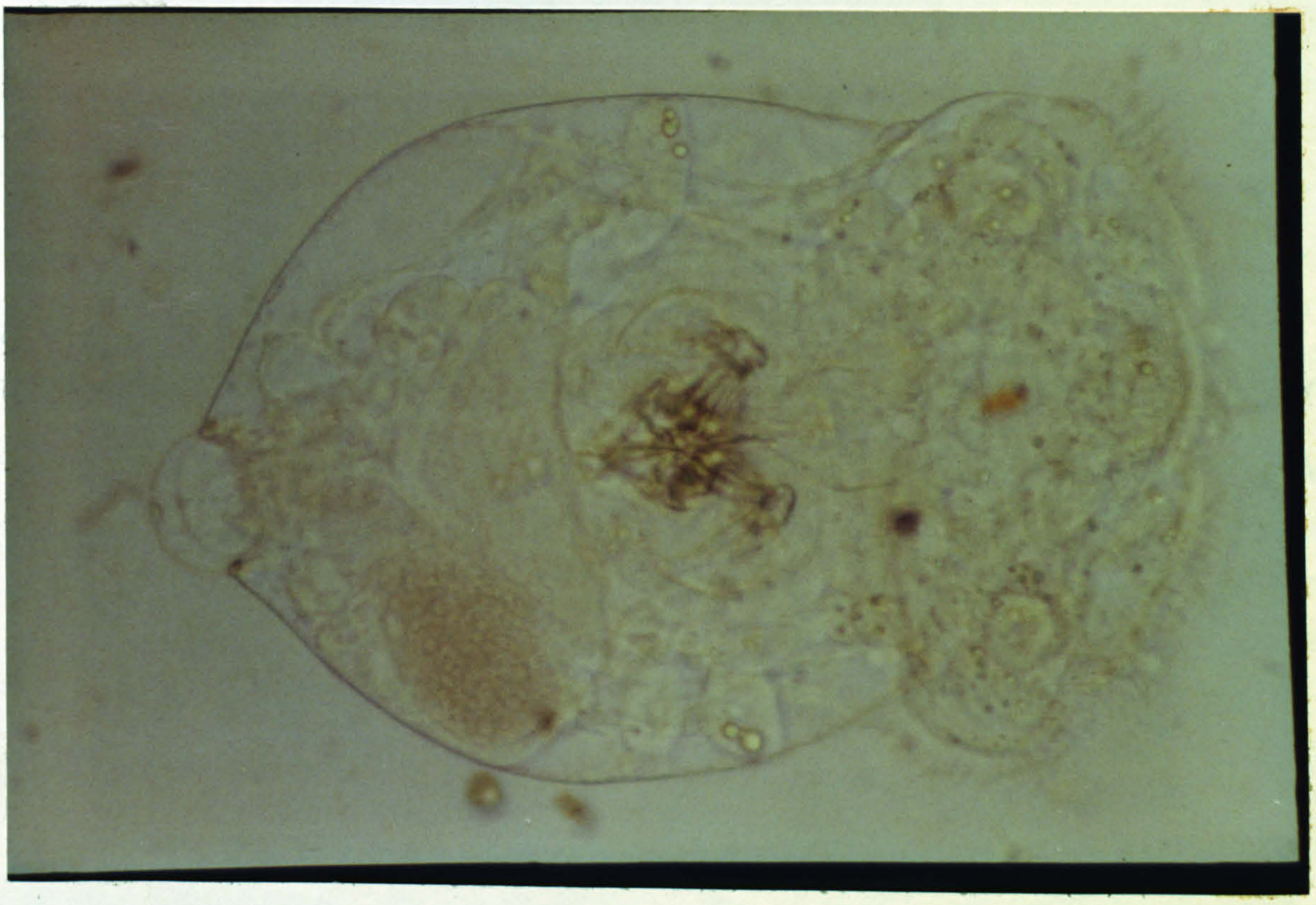

15
14
13
12
0
Length (mm)
a
a
b
40
35
30
25
0
Weight (mg)
a
b
c
Length
Weight
= control
= CAR
= Booster

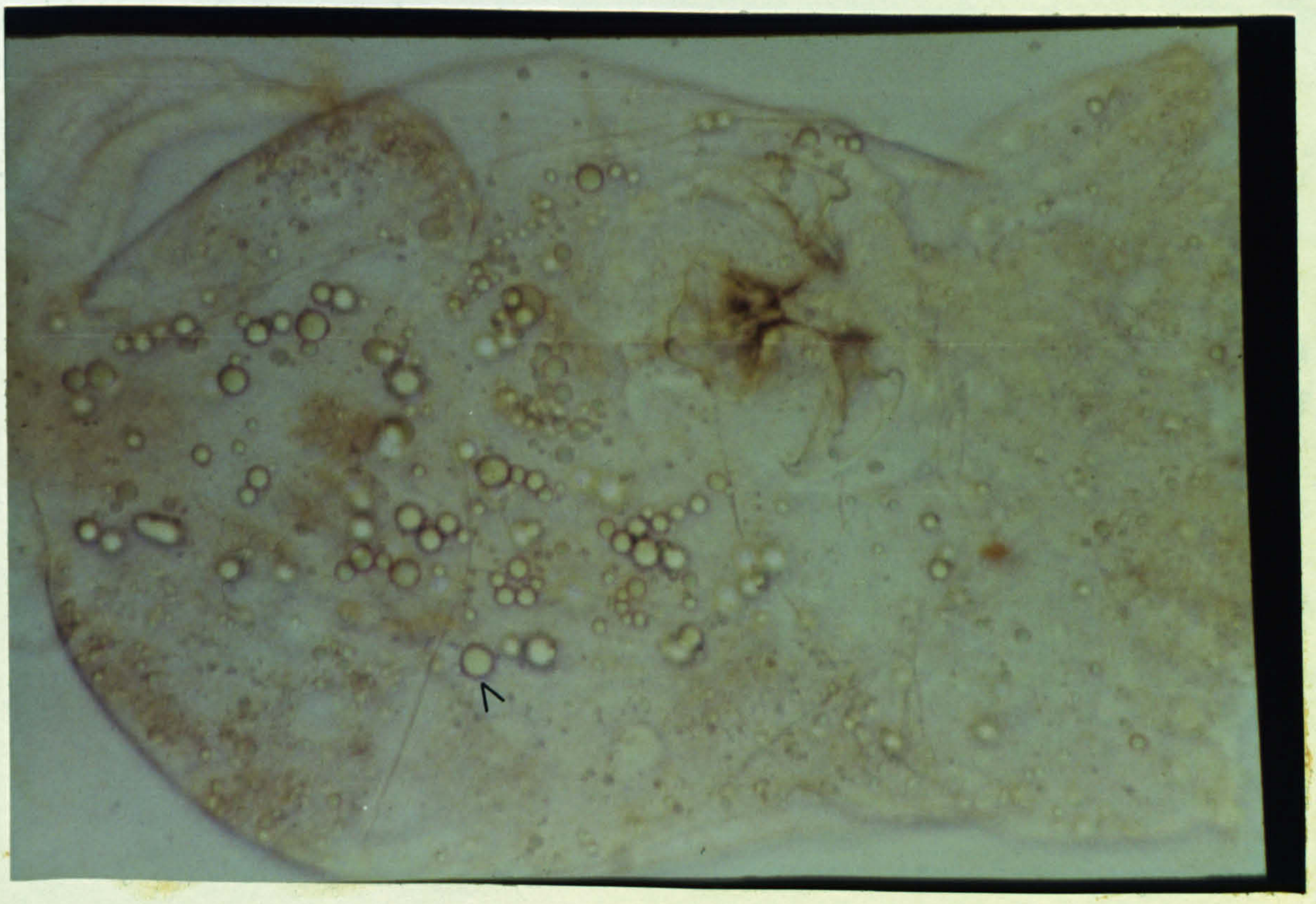
Plate 1. Photomicrographs of examples of rotifers after 4h feeding feeding on Frippak CAR (A) and after 4h feeding on Frippak Booster (B) showing accumulation of lipid droplets (arrow).

⊢——⊣ = 20 μm

(A)



(B)



Chapter 3

GROWTH, SURVIVAL, FEEDING AND DIGESTIVE ENZYME ACTIVITY IN CARP (CYPRINUS CARPIO) LARVAE FED ARTEMIA NAUPLII OR A SPRAY-DRIED MICROENCAPSULATED DIET.

Parts of this chapter have contributed to:

Le Vay, L. and Jones, D.A. (1992) Digestive activity in carp larvae feeding on Artemia and artificial diets. Poster presentation in Experimental Aspects of Aquaculture Symposium, Society for Experimental Biology, Lancaster University, April 1992.

Le Vay, L., Jones, D.A. and Guzmán, J. (1993). Microencapsulated diets as feeds for fish larvae: problems and prospects. Proc. 9th Int. Microencapsulation Symp., Ankara, Turkey, Sept. 1993 (in press).

INTRODUCTION

The commercial culture of crustaceans, fish and bivalves is largely dependent on the rearing of larvae using live natural foods (review Chapter 1). This is particularly true in the case of fish larvae, for which the routine total replacement of live food remains elusive. Both microbound and microencapsulated particles have been used as start-feeds for fish larvae, the microbound forms producing the best results so far, at least with the relatively large larvae of freshwater species (see Table 2, Chapter 1). Microencapsulation still offers the best potential for development of very small diet particles for early marine fish larvae, due to their relatively high physical and chemical stability and smooth surface texture. Since the development of microencapsulated diet particles (Jones et al. 1987) the commercial-scale culture of penaeid shrimp larvae with almost complete replacement of live food has become possible.

In contrast to shrimp larvae, early fish larvae ingest particles whole and in most marine and freshwater species have a poorly developed digestive system. It has been suggested that some autolysis of live prey within the larval fish gut may be essential to promote efficient digestion (Dabrowski and Glogowski 1977a, Munilla-Moran et al. 1990), although there is also evidence that some secretion of proteases does occur in response to ingestion of inert particles (Hjelmeland et al. 1988, Rösch and Segner, 1990, Pedersen and Andersen 1992). However, any such activity appears to be insufficient to break down the wall of cross-linked protein microencapsulated feeds, which have been observed to remain intact within the gut of larvae of some species (Kanazawa et al. 1982b, Walford et al. 1991). For this reason, attempts to totally replace live food for fish larvae with cross-linked protein microencapsulated diets have been unsuccessful. When rotifers were fed to Lates calcarifer larvae together with cross-linked protein microcapsules survival and growth equivalent to, or better than, live food treatments alone was achieved (Walford and Lam 1991). The presence of live rotifers in the gut improves the breakdown of microcapsules and is attributed to the supply of exogenous enzymes by prey (Walford et al. 1991), though live food may also stimulate digestive enzyme secretion by fish larvae (Munilla Moran and Stark 1989). Vergara-Martin et al. (1990) report the successful use of a spray-dried microencapsulated diet as a 50% replacement for rotifers in the rearing of Sparus aurata larvae, although larvae did not survive when rotifers were totally replaced by the artificial diet.

As well as problems associated with digestibility of diet particles, fish larvae are primarily visual feeders (Blaxter and Staines 1971) although they do display some chemosensory ability to locate prey patches (Dϕving and Knutsen 1993). Successful

presentation of diet particles appears to be dependent on providing movement, usually achieved by wet or dry drip-feeding of particles at the water surface and allowing them to fall through the water column. However, such methods often produce less than satisfactory levels of ingestion of artificial diet particles - particularly by early larvae (Fox 1990).

In the present chapter, carp larvae were used to test the suitability of a spray-dried microencapsulated diet as a start-feed for fish larvae. Measurements were also made of feeding rates and digestive enzyme activity in carp larvae offered either live food or the artificial diet. These experiments provide the basis for discussion on potential areas for improvements to microencapsulated diets for fish larvae.
The specific objectives of the study are outlined below.

1. As a preliminary to feeding experiments with larvae, the nutritional suitability of the diet for post-larval carp is examined.

2. The survival and growth of larvae fed live and artificial diets are investigated, using two different methods of diet delivery.

3. Observations are made on stability of the artificial diet within the larval gut.

4. Ingestion rates of live and artificial diets by first feeding larvae is quantified by direct observation of feeding behaviour, gut emptying rates, gut prey content and by a gravimetric method.

5. A study is made of proteolytic enzyme responses by carp larvae, at various stages of development, to live and artificial diets.

6. The results of the above experiments are used to estimate energy assimilation efficiencies for first-feeding larvae ingesting live food or the artificial diet.

7. A novel form of cross-linked protein microencapsulated diet particle is tested for stability in vitro and within the larval gut.

GENERAL MATERIALS AND METHODS

Experimental recirculating water system

A recirculating water system was constructed, as illustrated in Figure 1. The system, which has a total capacity of 500 l, was filled with tap-water. Water was pumped from the sump tank through 100 μm and 50 μm cartridge-filters and a U.V. unit to the header tank, where it was heated by a 1 kW submersible element controlled by a digital thermostat. The large capacity of the system enabled temperatures to be maintained within $\pm$ 0.1 ^{o}C during experiments. Aeration was supplied from the

main laboratory system through airstones in both the sump tank and the header tank. Water left the header tank either though a gravity-fed spray bar to either a bench-top raceway or though a series of taps to individual larval rearing containers standing in the raceway. Overflow from the raceway was returned to the sump tank after passing through a terylene wool filter and a sedimentation tank. Water was continuously pumped over a biological trickle filter on a side circuit from the sump tank. By use of a siphon valve on the outflow of the raceway it was possible to regulate a cycle of partial filling and emptying of the raceway providing efficient exchange of water in mesh-bottom larval rearing containers. When larvae were maintained in 2 l round-bottom flasks, the raceway provided a constant temperature water bath and daily exchanges were made using water from the recirculation system.

Supply of carp eggs and larvae

Batches of mirror carp (Cyprinus carpio) eggs were supplied by Munton and Fison PlC, Stowmarket, and hatched as described in Chapter 2. Larvae were transferred to experimental treatments once they had developed open mouths and inflated swim bladders. Stock groups of fish were maintained in the hatching tank and fed freshly hatched Artemia nauplii.

Artemia

Cysts of Artemia (Artemia 90 brand, Sanofi Aquaculture) were hatched overnight in 2 l flasks, at a density of 4g of cysts per flask, under strong aeration and illumination in U.V.-irradiated filtered seawater which had been adjusted to 15 ^{o}/oo salinity with distilled water. After approximately 16 h, aeration was stopped to allow unhatched cysts to sink, empty cyst cases to float and nauplii were collected from the middle of the flask by siphoning. Nauplii were separated from any remaining unhatched cysts by concentration to a point light source in a dark plastic tray. 1 ml samples of the concentrated nauplii were diluted x 100 in cold distilled water, and their density was estimated by counts of 1 ml sub-samples from the diluted suspension. Where several feeds of Artemia were required each day, nauplii from overnight hatching were kept at 4 ^{o}C under gentle aeration until needed.

Artificial diets

Spray-dried microencapsulated diets were supplied by Frippak Feeds, Aberdeen. For larval feeding trials, the Frippak CD3 range were used. These diets are produced as an Artemia replacement for the carnivorous larval stages of penaeid shrimp, with a particle size range of mode 150 μm, median 137μm. Composition of the diet was 53% protein, 20% lipid, 15% carbohydrate/fibre, 12% ash (data supplied by Frippak). In order to measure soluble protein content of the diet, samples were

homogenised in distilled water, centrifuged and the supernatant assayed for protein using the Sigma protein test kit (modification of the Lowry method by Onishi and Barr 1978). 0.1 ml of supernatant was mixed with 1.0 ml of Biuret's reagent and left to stand. After 10 min, 0.05ml of Folin's reagent was added and absorbance at 600nm read after a further 30 min. Readings were calibrated against a standard curve prepared with bovine serum albumin.

For post-larval feeding experiments a pan-granulation method was used to form aggregations of CD3 diet (particle size ranges from 200 to 600 μm) with a cellulose binder (performed by Frippak Feeds, Findon Laboratory, Aberdeen).

Trypsin assay

Samples of larvae, Artemia and CD diet were homogenised in ice-cold Tris-HCl buffer (46 mM Tris, 11.5 mM $CaCl_2$, pH 8.1). In the case of post-larvae, gut tissue was excised and used in enzyme assays. Homogenates were centrifuged at 8,000 x g for 5 min and the supernatant was assayed for trypsin-like activity at 25°C using either 1 mM Nα-p-toluenesulphonyl-L-argenine methyl ester (TAME) (Hummel 1959) or 1 mM Nα-benzoyl-L-argenine-p-nitroanilide (BAPNA) (Sarath et al. 1989). TAME stock solution was prepared in the same tris buffer. BAPNA was dissolved in 2ml of dimethyl sulphoxide, and made up to 10ml with 50% methanol in water. In both cases 100μl of substrate solution and 700μl of buffer were equilibrated at 25°C in 1.5ml Eppendorf tubes. 200μl of sample was added, well mixed, and the reaction mixture transferred to a 1 cm path-length quartz cuvette. In the case of samples assayed with TAME, the cuvette was placed in a thermostatically-controlled holder maintained at 25°C in a Hewlett Packard 8452A Diode Array spectrophotometer. Absorbance at 247 nm was logged on a Hewlett Packard Vectra E5/12 microcomputer, every 6s for 180s. In the case of samples assayed with BAPNA, increase in absorbance at 405 nm was measured in the same 1 cm path length quartz cuvette, in a thermostatically -controlled hold, in a Cecil CE 1010 spectrophotometer connected to a desk-top computer which operated the spectrophotometer and a data logging programme. In all cases triplicate assays were performed on each sample. Trypsin activity in the sample homogenate was calculated as follows, and expressed in units of μmol of substrate cleaved per minute.

$$\text{Total activity in sample} = \frac{\text{sample vol. x assay vol.}}{\text{vol. of sample in assay x } \epsilon \text{ x d}} \times \delta Abs/\delta t$$

ϵ = extinction coefficient (= 0.54 cm^2 μmol^{-1} for TAME, 9.62 cm^2 μmol^{-1} for BAPNA)

Abs. = absorbance, t = time (min), d = path length (cm)

Total protease assay

Total protease activity was assayed using casein as substrate in a method adapted from Kunitz (1947). Samples were prepared as described for trypsin assay, in the same Tris buffer, except that pH was adjusted to 7.5. A mixture of 100 μl of sample supernantant and 400μl of casein solution in buffer was incubated in Eppendorf microcentrifuge tubes for 60 min. The reaction was stopped by the addition of 1 ml of ice-cold 0.6 M trichloroacetic acid. Precipitated protein was removed by centrifugation, and the absorbance of the supernatant measured at 280nm against enzyme and reagent blanks. The increase in absorbance was calibrated against a tyrosine standard, and one unit of activity was defined as that amount of enzyme which releases 1 μg of tyrosine in 1 min under the assay conditions used.

Preparation of cross-linked protein microcapsules

Microcapsules were prepared from either cod roe or haemoglobin using the method of Jones et al. (1987). Frozen cod roe was homogenised in a Silverson blender, diluted 25% with distilled water and passed successively through 250μm, 140μm and 45μm sieves. Haemoglobin was prepared as 25% w/v solution in distilled water. 50ml of cyclohexane containing 2% w/v lecithin was placed in a conical flask with a magnetic stirring flea. With the stirrer at half speed, 5ml of the cod roe homogenate or haemoglobin solution was added dropwise over several minutes and the stirrer speed was adjusted to achieve dispersion of fine droplets. 0.2ml of a cross-linking agent (succinyl dichloride) was dissolved in 10ml of cyclohexane, prior to addition to the reaction flask. After a fixing period of between 6 - 12 min, the reaction was stopped by quenching with 50 ml of cyclohexane. The stirrer was stopped, and microcapsules allowed to settle to the bottom of the flask. The cylcohexane was decanted, and replaced in two washes with clean cyclohexane. After the final wash with cyclohexane, the microcapsules were collected and poured into a glass petri dish and allowed to air dry for 5 min in the fume cupboard. Once all visible cyclohexane had evaporated, the microcapsules were washed twice in a Tris-HCl buffer (pH 8.5), followed by a final wash in distilled water. At this stage, microcapsules were either stored wet at 4^{o}C until used the same day, or freeze dried overnight prior to storage at -20^{o}C.

Cod-roe microcapsules were also prepared with the addition of between 0.025% to 1% (w/v) porcine pancreatin to the homogenate. Haemoglobin microcapsules were prepared after addition of 1% to 10% (w/v) pancreatin to the protein solution.

EXPERIMENTAL METHODS

Animals (Scientific Procedures) Act

Use of anaesthetic (MS-222) to sedate and restrain fish larvae (for microscopical examination or weighing) once they are capable of independent feeding brings such experiments into the 'protected' category of the Animals (Scientific Procedures) Act 1986. All such treatments were carried out by Dr. D.J. Grove (PIL 40/01630, PPL 40/01096) in a Designated Scientific Procedure Laboratory.

Growth of carp postlarvae fed artificial diet or Artemia nauplii

In order to test the nutritional suitability of the artificial diet formulation for young carp, the growth of post-larval carp fed a diet formed by pan-granulation of Frippak CD3 was compared to that of postlarvae fed Artemia nauplii. The aim of the trial was to compare growth rather than survival, as results in the literature suggest that low mortality might be expected in post-larval carp weaned to artificial diets at initial weights of more than 15mg (Bryant and Matty, 1981). Larvae were reared in stock-holding aquaria as described above and fed Artemia. 10 days after first-feeding, fish of approximately 40mg wet weight were allocated to the dietary treatments. As growth rate of carp larvae fed artificial diets may be dependent on weight at weaning (Bryant and Matty, 1981), the pool of fish used in the experiment was selected to be of approximately similar initial length and weight, as judged by eye. 15 fish were selected for each container, with care being taken to ensure consistent size of fish across all treatments. The combined weight of all fish in each group was recorded. Each group was placed in an experimental container, and three groups randomly allocated to each treatment. Every two days all the fish were removed from each container, anesthetised with MS-222 and the total length of each fish and the weight of the group was recorded. Removal of the fish also allowed thorough cleaning of the containers.

For each treatment a stock of fish was maintained in 10l aquaria under the same feeding regime. When mortalities occurred in the experimental treatments, the lost fish were replaced with fish of similar size from the appropriate stock tank. During the experiment, only four mortalities were recorded.

Experimental rearing containers were flat-bottomed, white, polythene buckets. Each container had a rectangular outflow cut around half its circumference, which was covered with a 200μm mesh to prevent escape of diet or Artemia nauplii. Water volume in each container during the experiment was approximately 2000 ml. Water flow to the containers from the header tank was through a grid of 20mm PVC piping, with plastic air line used to provide a small bore outflow pipe to each tank. The flow

rate to individual containers was controlled by clamps on each outflow pipe, and maintained at approximately 100 ml minute^{-1}. The containers were placed in the raceway and all outflow water returned to the recirculating system. The temperature in the water system was maintained at 24 $\pm$ 1^{o}C.

The artificial diet was offered at a rate of 40% body weight day^{-1}, based on the previous weight estimate for each container. Freshly hatched Artemia were added to the live feed treatment at a rate equivalent to 100 nauplii/mg fish body weight per day, based on the previous weight estimate. For both treatments, feeding was carried out five times during each day at 4h intervals. After each feed the water supply to the experimental containers was turned off for 30 min to prevent clogging of the outflow mesh with food material. Faeces and waste food were siphoned from the bottom of each container three times daily.

Growth and survival of larvae fed artificial diet or Artemia

Two experiments were carried to examine the growth and survival of carp larvae when fed the artificial diet, in comparison with larvae fed Artemia nauplii. In a first experiment, groups of 50 larvae were transferred to experimental treatments on the day after swim-bladder inflation was first observed. Larval rearing containers were 20cm lengths of ABS drain-pipe, 16 cm diameter, fitted with mesh bottoms (700μm for larvae fed artificial diet, 180μm for larvae fed Artemia or unfed controls). The containers were fitted with feet (1cm depth) to facilitate water exchange as the level in the raceway changed. The water inflow to the raceway was adjusted to maintain a filling and emptying cycle which produced 25% exchange of water 5 to 6 times per hour. The volume of water in the larval containers ranged between 1.5 litres to 2 litres during the cycle. The initial total length of 20 larvae was measured using a binocular microscope fitted with a calibrated graticule. Three groups of 50 larvae were allocated to each dietary treatment. Artemia nauplii were fed to the control groups in five feeds per day, each equivalent to 5 nauplii ml^{-1}. Artificial diet was administered dry to the water surface in five feeds per day, from 0800 to 2400 (initial daily ration 100mg/container). The ration was increased as the larvae grew to ensure excess food throughout the experiment. Prior to each feed the experimental containers were cleaned by siphoning and any mortalities recorded. On the 6th day from the start of the experiment total lengths of samples of 10 larvae from each container were measured. On the 11th day from first-feeding the experiment was terminated, all fish were killed with excess MS-222 and the weights and lengths of all fish recorded.

During the experiment, the gut fullness and feeding behaviour of larvae under conditions identical to those used for the growth experimental treatments was

observed. It was noted that most of the artificial diet tended to remain on the surface of the water immediately after feeding and slowly fall to the bottom over the subsequent 2 - 3 h. During the first six days of feeding, larvae were observed during approaches to diet particles at the surface, and feeding behaviour was recorded as;

1. Encounter: Food particle investigated but not taken into mouth.
2. Accept: Food particle taken into mouth and swallowed.
3. Reject: Food particle taken into mouth but rejected.

Following the results of the above experiment, a second experiment was conducted in which larvae were reared in round-bottom 2 litre flasks. Gentle aeration was supplied through a glass pipette, and each day larvae were transferred to clean water in clean flasks. The same feeding regimes and stocking densities were used as in the previous experiment, except that the artificial diet was hydrated in distilled water for 10 min prior to addition to the flasks. Visual observation of larvae feeding in flasks indicated that food ingestion was much greater than when diets were fed to the surface of containers used in the previous experiment. This was due to larvae feeding readily on diet particles kept in suspension by aeration and on diet particles resting on the sloping sides of the flasks.

Quantification of the transit rate of Artemia and the artificial diet through the gut of first-feeding larvae in flasks

In order to follow the passage of food through the guts of first feeding larvae, colour-marked forms of the diets were prepared. Artemia nauplii were marked by pre-feeding on fine carbon particles for one hour prior to being fed to the carp larvae. The artificial diet was marked by hydrating in a red domestic food dye (carmoisine artificial cochineal). In both cases the marked diets were fed to larvae under similar conditions as used in the second growth trial (in flasks). Once the larvae had been feeding steadily on the marked diets for one hour, they were carefully transferred to clean water in new flasks to which unmarked Artemia or artificial diet were added. At ten min intervals, five larvae were sampled from the flasks and examined under a binocular microscope. The position of the boundary between the marked and unmarked food was recorded as a percentage of the length of the gut between the front of the swim bladder and the anus.

Quantification of gut content of first-feeding larvae

The quantity of diet ingested by first-feeding larvae in flasks was estimated by a gravimetric method. Groups of 2 mg and 4 mg larvae were placed in 2 l round-bottom flasks and fed either Artemia nauplii or the artificial diet. At the same time up to 20 individual unfed larvae were sampled, killed with excess MS-222 and laid out in

rows on an aluminium foil sheet. After 3 h feeding, larvae were sampled from each treatment and placed on foil sheets. All larvae were dried to constant weight at 80^{o}C and stored in a desiccator. Small pieces of foil, carrying individual larvae, were cut out from the foil sheets and weighed on a Cahn C-31 electronic microbalance. Immediately after weighing, the larva was carefully removed from the foil by scraping with a blunt point, and the foil re-weighed. Dry weight of gut food content was calculated as the difference between the mean weights of fed and unfed groups, and a 95% confidence interval for the difference was obtained by a two sample t-test in Minitab.

Fish may compensate for a period of starvation by hyperphagy on encountering a high density of prey, and thus a comparison of the previous gut content estimates with those of larvae under continuous feeding conditions was necessary. Larvae were sampled from flasks in which larvae had been continuously feeding on Artemia. Sampled larvae were killed with excess MS-222, examined under a binocular microscope and the number of Artemia within the gut recorded. Using a value of 2μg dry weight per nauplius (averaged from Benijts et al. 1975), the weight of food in the gut was calculated. This approach was not applied in the case of larvae feeding on the artificial diet, but the ratio between values from the two methods for larvae feeding on Artemia provided a correction factor for estimation of gut contents of the artificial diet under continuous-feeding conditions.

Trypsin and general protease activity in larvae and post-larvae following weaning onto artificial diets from Artemia nauplii

Carp larvae were reared on Artemia from first feeding in the stock-holding aquaria, as described above. At weights of 1.5mg - 95mg, groups of larvae were transferred to round-bottom flasks and fed either artificial diet or Artemia nauplii. Several samples of 20 larvae were collected from each of the experimental treatments after 24H, killed with excess MS-222 frozen and stored at -20^{o}C. Trypsin activity in samples was assayed using TAME and general protease using casein, as described earlier.

Trypsin activity in first-feeding larvae fed artificial diet or Artemia

In a second experiment, changes in trypsin activity in 2mg and 4mg larvae during feeding on Artemia nauplii or artificial diet were examined. Larvae (wet weight approximately 2mg) which had been fed Artemia for 24 h since initiation of feeding were transferred to clean flasks and fed artificial diet. At the same time, 2mg larvae which had been fed the artificial diet for 24 h since initiation of feeding were transferred to clean flasks and fed Artemia nauplii. Another group of larvae was taken from the stock-holding aquaria 72 h after initiation of feeding on Artemia nauplii. These larvae (wet weight approximately 4 mg) were transferred to round-

bottom 2 l flasks and fed the artificial diet. A series of samples of 10 larvae were collected from all three groups of larvae over the 10 h following transfer to the new diets. Samples were killed with excess MS-222 and stored frozen at -20^{o}C until assayed for trypsin activity using BAPNA as substrate.

During the above experiments larvae were also removed from each treatment, killed with excess MS-222, and examined under a binocular microscope. The position of the boundary between the two food types along the gut was quantified as a percentage of the length of the gut between the front of the swim bladder and the anus. The time taken for the gut to become completely full of the second food type was recorded.

Estimation of endogenous trypsin activity in early larvae.

Results from the previous experiments indicated significant differences in trypsin activity in whole-fish homogenates of larvae fed Artemia or the artificial diet. However, measurement of trypsin activity in samples of Artemia nauplii and of the artificial diet suggested that exogenous trypsin activity might contribute a large proportion of total trypsin activity. In order to estimate what proportion of the measured activity was endogenous, and thus a direct response to diet, calculation of potential enzyme contribution from the two diets was necessary. Samples of freshly hatched Artemia nauplii and the Frippak diet were assayed for trypsin activity using the BAPNA method described earlier. A range of values for contribution of exogenous trypsin from the two foods was calculated based on the 95% confidence ranges for gut content (corrected values for continuously feeding fish) and total larval trypsin activities. Exogenous activity was subtracted from the total activity recorded for larvae to give an estimate of endogenous larval trypsin activity.

Stability of cross-linked protein microcapsules

The in vitro stability of cross-linked protein microcapsules was tested by visual examination after hydration in seawater at 20^{o}C. Capsules were suspended in a 10 ml of seawater, and placed in a glass petri-dish on a heating block set at 20^{o}C. At hourly intervals after hydration small samples of the capsules were removed with a Pasteur pipette and examined under a compound light microscope.

Both types of cross-linked microcapsule were offered to first-feeding carp larvae in round-bottom flasks. Haemoglobin microcapsules containing 5% pancreatin were also fed to first-feeding carp larvae together with the Frippak CD3 spray-dried diet. In all cases, larvae were removed and killed at hourly intervals and stability of food particles within the gut examined under a compound light microscope.

RESULTS

Growth of postlarvae fed artificial diet or Artemia nauplii

The pan-granulated form of the artificial diet was clearly highly attractive and acceptable to young mirror carp and was readily consumed. The artificial diet appeared to be highly stable in water, with no apparent loss of water quality in the experimental system despite the high feeding rates used.

Prior to statistical analysis of growth data, linearity of length and weight against time was tested using the runs and sign tests facilities in the Fig-P/P-fit graphics and curve-fitting software package (Biosoft, Cambridge). Weight data were transformed to natural logarithms to achieve linearity, while length data were considered linear. Mean lengths pooled from the three replicates and ln mean group weights are shown in Figure 2, together with regression lines fitted to ln weight data and to pooled length data. The slopes of the ln weight plots correspond to the specific growth rate, defined as;

$$S.G.R. = 100 \times [(\ln W_T - \ln W_t)/(T - t)] \% D^{-1}$$

Where; W_T = weight at start and W_t = weight at end)

Thus in the present study, the specific growth rate of post-larvae carp fed Artemia was 27% (± 95% C.I. of 2%), while that of fish fed the artificial diet was 16% (± 95% C.I. of 3%). Analysis of covariance of length and ln transformed weight data with treatment, replicates nested within treatment and with time was carried out using Minitab (version 6). Results (Appendix 3) show that there was no significant difference in growth rates between replicates, and that differences in growth rate between treatments, in terms of both length and weight, were significant ($p<0.001$).

Growth and survival of larvae fed artificial diet or Artemia nauplii

The survival and growth of carp larvae fed Artemia or the artificial diet in the first experiment (open containers) are shown in Figures 3a and 3b. Survival of larvae fed Artemia was close to 100%, while over 60% of larvae fed the artificial diet had died at day 3, with final survival (day 11) of 5%. Final weight of larvae reared on Artemia was significantly higher (mean = 40mg) of those fed the artificial diet (mean = 2.6mg) (data pooled from replicates, $t=20.39$, $p<0.0001$). Growth in terms of length was linear throughout the experiment for larvae fed Artemia, while those fed the artificial diet showed markedly less growth from day 6 to day 11 than in the first half of the experimental period (Figure 3b). At day 6, larvae fed Artemia were already significantly longer than those fed the artificial diet (nested ANOVA, $F=241.7$, $p<0.001$).

Despite the improved ingestion of the diet in flasks, larval growth and survival was poor. Results of the second growth trial (flasks) are shown in Figure 4. Again

survival of larvae fed Artemia nauplii was excellent, only falling to 92% at day 5 with no further mortality thereafter. Larvae fed the artificial diet exhibited similar survival until day 4, but between day 4 and day 6 survival fell to 28%, with final survival of 15% at day 10. At the end of the experiment larvae reared on Artemia were significantly heavier (34.5 ± 8.3mg) than those reared on the artificial diet (5.7 ± 2.4 mg) (data pooled from replicates, $t=14.06$, $p<0.0001$). Larvae reared on Artemia were significantly longer (15.3 ± 2.5mm) than those reared on the artificial diet (9.48 ± 1.1mm) (data pooled from replicates, $t=11.38$, $p<0.001$).

Ingestion rates

Observation of larvae feeding under the same conditions that applied in the first larval rearing experiment (open containers) indicated very poor ingestion of the artificial diet. Figure 5 shows results of observations of larval investigation of diet particles at the water surface. On the first day of feeding only 17% of investigations of diet particles on the surface were followed by ingestion. In 45% of cases the particle was approached but not taken into the mouth ('encounter') and in 38% cases particles were taken into the mouth but rejected ('rejection'). During the following two days the percentage of 'encounters' fell to 5-8%, while the incidence of ingestion rose to 33%. However, over the first six days of feeding, ingestion did not rise above 38%, with the incidence of 'encounters' increasing again to 25% of investigations from day four to day six. Overall feeding rate was very low, compared to the live food treatment, and even in larvae which were feeding relatively well on the artificial diet, less than than 30% of total gut length was filled.

In contrast to these observations, larvae fed Artemia exhibited a high feeding rate, and rapidly filled their guts from first feeding. The diet sorting behaviour observed in larvae feeding on the artificial diet was not observed in larvae fed Artemia, with rapid ingestion of all nauplii which were approached.

Larvae offered the artificial diet in flasks were observed to feed readily on particles moving in the water and on particles resting on the sloping sides of the flasks. Even in the first few hours of feeding on day 1 larvae were observed with guts full of the artificial diet. For larvae feeding in flasks there was no significant difference between the gravimetric estimates of gut food content for larvae fed Artemia or the artificial diet, at either 2mg or 4mg wet weight. The estimated 95% confidence range for gut contents of 2mg larvae was 52 - 120 μg of the artificial diet compared to 75 - 147 μg of Artemia. The 4 mg larvae were found to have consumed approximately twice the amount of both diets as the 2 mg larvae, with again no significant difference between gut content of larvae fed the artificial diet (149 - 283 μg) or Artemia nauplii (121 - 262μg) (data in Appendix 3).

Counting Artemia nauplii in the gut of continuously feeding larvae yielded a lower estimate for weight of food ingested (mean 58.6μg, 95% confidence range 46.3 - 70.9, n = 20) than the gravimetric method. Accordingly a correction factor was calculated as;

mean weight of Artemia (counts) ÷ mean weight Artemia (gravimetric)

= 58.6 μg / 111 μg = 0.528

In the absence of any other means of measurement, it was assumed that the amount of artificial diet in the gut of previously starved fish would similarly overestimate the gut content of continuously feeding larvae. Applying the conversion factor derived from data for larvae fed Artemia yields an adjusted mean content of the artificial diet in continuously feeding 2 mg larvae of 45.6μg (95% confidence interval 27.6μg - 63.6μg).

Gut clearance data (shown in Figure 6) was linear against time, as determined by examination of residuals from regression analysis.

Fitted regression equations for the two food types were:

For Artemia

% gut length = 98.1 - 1.04 min (r^2 = 79.3%, F = 150)

For artificial diet

% gut length = 93.5 - 1.00 min (r^2 = 67.8%, F = 83.3)

Analysis of covariance of boundary position with time and treatment showed no significant difference (F = 0.07, p = 0.795) between the slopes of the two regression lines. Taken with the results for estimation of gut contents, present results indicate the two diets were ingested at the same rate for first-feeding larvae. For these larvae, mean daily intake of Artemia can be calculated as 879μg with a value of 587μg for the artificial diet.

Protease activity in larvae and post-larvae following weaning onto the artificial diet from Artemia

Figure 7a show results of trypsin assays for larvae from the three dietary treatments. In first feeding larvae (1 to 2 mg weight), trypsin activity was significantly higher in larvae fed Artemia nauplii than in either those fed the CD diet or unfed larvae ($p<0.05$). In 4 to 5 mg and 5 to 6 mg size ranges, significantly higher trypsin activity was recorded in larvae fed the artificial diet than those fed Artemia ($p<0.05$), with the unfed group yielding significantly lower trypsin activities than either of the fed treatments ($p<0.05$). In the 9 to 10 mg size range, trypsin activity in fish from the artificial diet treatment was lower than in the previous size ranges in

the same treatment, and there was no significant difference in trypsin activity in larvae from the three treatments. In the 12 to 14 mg size range, in which the carp were early postlarvae, high trypsin activities were recorded in fish fed either Artemia or the artificial diet, with significantly lower activity in unfed fish ($p<0.05$).

Trypsin activity in postlarvae fed Artemia or the pan-granulated artificial diet are shown in Figure 7b. In both treatments there was a linear increase in trypsin activity with fish weight. Linear regression equations fitted to the trypsin data are shown in Figure 7b, and have slopes of 0.01 Units mg^{-1} for fish fed Artemia and 0.017 Units mg^{-1} for fish fed the artificial diet. Analysis of covariance (Appendix 3) shows that there was a significant difference between the slopes of the two lines ($p = 0.025$), which both had y-axis intercepts very close to zero, and thus a significant difference in trypsin activity standardised against fish weight for the two treatments.

The pattern of general protease activity in larvae fed Artemia or the artificial diet was similar to that recorded for trypsin (Figure 8a). There was a good correlation between trypsin activity and protease activity for larvae of less than 12 mg ($r = 0.87$, Figure 8b), with a significant linear regression of trypsin activity against protease activity (slope = 10.61, intercept = 2.20, $p<0.000$). However, for larvae/postlarvae greater than 12 mg the high levels of trypsin activity recorded do not conform to this relationship (see Figure 8b), indicating that following metamorphosis trypsin may become a relatively more important component of total proteolytic enzyme activity.

Trypsin activity in early larvae fed artificial diet or Artemia

Figure 9a shows trypsin activity in 2 mg larvae during the transition from Artemia to the artificial diet and vice-versa. Initial trypsin activity in 2 mg larvae feeding on Artemia was significantly higher than in 2mg larvae feeding on the artificial diet ($p<0.05$). There was no significant difference in the rate of transit of the food boundary through the gut in 2 mg larvae transferred to the artificial diet ($1.10 \pm 0.05\%$ min^{-1}) and 2 mg larvae transferred to Artemia (1.12 ± 0.15 % min^{-1}) (t -test, Appendix 3). The gut of fish from both treatments had completely filled with the second diet after 90 min, during which time there was a significant rise in trypsin activity in larvae changing from artificial diet to Artemia. Thereafter, trypsin activity continued to rise over the 10 h following the transition from the artificial diet.

During the first 2 h of the transition from Artemia to artificial diet, activity dropped, but not significantly. After 4 h feeding on the artificial diet trypsin activity had dropped further to levels similar to those observed in the larvae initially feeding on the artificial diet.

Figure 9b shows trypsin activity in 4 mg larvae weaned from Artemia to the

artificial diet. There was no significant change in trypsin activity during the first 2 h. The gut of larvae was found to be completely full of the artificial diet approximately 4 h after weaning. At 5 h after weaning, trypsin activity had risen significantly compared to inital values ($p<0.05$), and continued to rise over the following 5 h.

Estimation of endogenous trypsin activity in early larvae.

Trypsin activity in Artemia was found to be $12.7 \pm 0.25 \times 10^{-3}$ Units mg^{-1} dry weight, compared to $2.57 \pm 0.45 \times 10^{-3}$ Units mg^{-1} dry weight for the artificial diet. Based on estimated gut food contents in 2 mg and 4 mg larvae and total trypsin values taken from the previous experiments (Figure 9), estimates of exogenous and endogenous trypsin activity were made as shown in Table 1.

In 2 mg larvae feeding on Artemia, exogenous trypsin contributed up to 15.5 - 24.1 % of total trypsin activity, while between 14.6 - 85.1% of total activity in 4 mg larvae feeding on Artemia might be attributable to diet. The artificial diet contributed between 47.% - 16.7,% of the trypsin activity measured in 2 mg larvae, and between 1.80% - 5.42% of activity in 4 mg larvae.

The estimated range of endogenous trypsin activity in 2 mg larvae fed Artemia nauplii (2.39 - 3.16 x 10^{-3} Units $larva^{-1}$) was higher than in 2 mg larvae fed the artificial diet (0.71 - 1.40 x 10^{-3} Units $larva^{-1}$). In contrast, the calculated range of endogenous trypsin activity in 4 mg larvae fed Artemia (0 - 4.47 x 10^{-3} Units $larva^{-1}$) was lower than that for 4 mg larvae fed the artificial diet (6.28 - 10.86 x 10^{-3} Units $larva^{-1}$).

In the second experiment, endogenous trypsin activity in 2 mg larvae fed the artificial diet was higher than, but overlapped with, the basal level of activity recorded in unfed larvae (Table 1). Thus the evidence for synthesis of trypsin in response to the artificial diet suggested by the total larval trypsin activities is less conclusive once the dietary trypsin contribution has been allowed for.

Assimilation efficiency

The observation of high ingestion rates implies that assimilation efficiencies, particularly of first feeding fish larvae, must be relatively low. Based on ingestion rates observed in the present study, together with growth rates of larvae grown in flasks, it is possible to make an estimate of efficiency of assimilation (as defined by Jobling, 1993) of dietary energy by first feeding carp larvae fed live and artificial diets, based on the formula;

$$K_A = \frac{(G \pm M)}{I} \times 100\%$$

where K_A = assimilation efficiency
G = growth (J)
M = metabolic energy consumption (J)
I = total ingested energy (J)

Measurements of growth and ingestion have been made in the present study. There is very good agreement in the literature on rates of oxygen consumption rates by early cyprinid larvae (Kaushik and Dabrowski 1988, Weiser et al. 1988, Kamler 1992) and a value of 1.5mg/g fresh weight/h is used here used to estimate metabolic energy use. Table 2 is a summary of the calculations made for first feeding carp larvae which show mean energy assimilation efficiencies of 12.9% for larvae fed the artificial diet and 19.1% for larvae fed Artemia. The value for the artificial diet may be overestimated as the calculation uses the specific growth rate of larvae surviving over 10 days. Those larvae which were unable to adapt to the diet sufficiently to survive might be expected to have grown more slowly from first feeding. For fish fed Artemia it is likely that growth rate at first feeding was higher than indicated by the specific growth rate calculated over 10 days, as specific growth rates of carp larvae fed zooplankton decline with larval age and are typically close to 50% over the first days of feeding (Bryant and Matty 1980, Kaushik and Dabrowski 1983). Hence assimilation efficiency over the first 24h would have been higher than the values calculated here, an error compounded by the fact that food supply probably had a limiting effect on growth towards the end of the trial (see discussion on growth rates above). Assuming a specific growth rate of 50%, the energy assimilation efficiency of larvae fed Artemia might have been as high as 26%.

Stability of the spray-dried artificial diet within the larval gut

During the larval rearing experiments, larvae were sampled while feeding on the spray-dried artificial diet and examined under a compound light microscope. Observations indicate that diet particles were physically stable within the larval gut, and passed through almost intact (see Plate 1). This was found to be the case not only for first feeding larvae, but even in some larvae after 6 days feeding on the artificial diet.

Stability of cross-linked protein microcapsules.

The general materials and methods section of the present chapter describes the preparation of cross-linked protein microcapsules from cod-roe and haemoglobin. As can be seen in Plates 2a and 3a, both types of capsule remained intact after 12 hours hydration in seawater at 20°C. Addition of pancreatin to the cod-roe at levels between 0.05% - 1% w/v produced microcapsules which auto-digested after 1-2h hydration in

seawater at 20°C (Plate 2b). The same capsules disintegrated within the gut of first-feeding carp larvae, while cod-roe microcapsules without pancreatin remained completely intact (Plate 4a,b).

Haemoglobin microcapsules containing 5-10% pancreatin were found to disintegrate within 1h in seawater at 20°C (Plate 3b) and also to digest rapidly within the larval gut (Plate 5a). When haemoglobin capsules containing 5% pancreatin were co-fed with the Frippak CD3 diet, visual examination suggested that particles of the spray-dried diet were physically broken down in the larval gut (Plate 5b).

DISCUSSION

Growth and survival of larvae and postlarvae

The specific growth rate of carp larvae reared on live food in the present experimental system is towards the low end of the range of values recorded in other studies (Table 3). However, growth of carp larvae can depend on feeding rate, temperature and on the nutritional quality of the Artemia strain used (Bryant and Matty 1980, Grove and Fletcher 1988, Kamler 1992), so that direct comparisons with other studies must be fairly circumspect. However, the feeding rate and rearing conditions used for larvae in the present study may not have been optimal, as the growth rate of postlarvae fed the same Artemia compares well with Bryant and Matty's (1980) best results for nine day old mirror carp larvae fed under very similar conditions. Specific growth rates calculated for larvae fed rotifers in Chapter 2 are superior to those recorded for Artemia-fed fish in the present study. However, it should be noted that the data for rotifer-fed fish is from an experiment in which larvae were stocked at half the density used in the present study. In particular, food availability may have been limiting towards the end of the experiment, when all Artemia were consumed during the periods between feeding times. Also the handling necessitated by water exchange may have had a negative effect on growth rate.

The excellent survival and good growth rate of post-larvae fed the pan-granulated Frippak diet suggests that the diet is nutritionally suitable for young carp. The observed specific growth rate was intermediate in the range of values calculated from published results, while survival of 96% compares favourably with the best results in the literature (see Table 4), despite the intense handling to which the fish were exposed during the experiment.

The growth and survival of larvae fed the artificial diet in open containers was very poor. On the evidence of visual observations this may largely be attributed to very low diet ingestion and poor diet digestibility. In the case of larvae fed the artificial diet in flasks, where ingestion was similar to that observed for live food, the

specific growth rate was at the low end of the range of published results for other artificial diets - though clearly not optimal (Table 4). Survival was poor compared to results in the literature, particularly where yeast-based diets have been used (Table 3). However, it should be noted that in the present study the use of flasks may have improved ingestion of the diet at the cost of the need for daily handling of larvae during water exchange. Furthermore the accumulation of diet and faeces in the static water during each day may have resulted in loss of water quality and reducing larval survival. Thus the low survival observed in the artificial diet treatment is likely to reflect the methodology of the experiment, over and above any nutritional deficiency of the diet.

Rate of food ingestion rate by first feeding larvae

The rate at which food is passed through the gut of fish larvae is dependent on feeding conditions. In the absence of further prey (i.e. after a single discrete meal) food is retained for longer in the gut than if feeding is continuous (Laurence 1971, Fänge and Grove 1979). When food is continuously available, feeding rate and consequent rate of food transit can be dependent on the density of prey offered (Werner and Blaxter 1980). In the present study, the rate of movement of food through the gut of first feeding larvae, with a total gut clearance time (GCT) of 100 min, is similar to values recorded for other early cyprinid larvae under continuous feeding conditions. Hofer and Nasir Uddin (1985) report a GCT of 2h for 2-3 mg Rutilus rutilis larvae fed Artemia. Kaushik and Dabrowski (1983) reported that 50% evacuation of food from the gut of first-feeding Cyprinus carpio larvae took 45 min after a single meal of zooplankton at 23^{o}C. In other freshwater species reported GCT values for continuously feeding early larvae are between 2h - 4h (Eldridge et al. 1982 for Morone saxitilis; Laurence 1971 for Micropterus salmoides). In comparison, GCT for continuously feeding marine fish larvae may be as low as 40 min or as high as 10h depending on species, food density, temperature and larval age (see Fange and Grove 1977, Govoni et al. 1986 for reviews).

In the present study, the dry weight of food in the gut of three day old carp larvae was higher when feeding resumed after a period of fasting than during continuous feeding. The amount of artificial diet retained after overnight starvation represented 19.8% - 45.6% of prefeeding body weight, while ingested Artemia represented 33.2% - 50.8% of body weight. These values compare with a range of 17.6% - 27.0% for larvae offered Artemia under continuous feeding conditions. It is likely that for post-fast larvae, the amount of food ingested represents close to the maximum gut capacity for larvae of this size, particularly in the case of larvae fed Artemia which had greatly distended guts. Expansion of the foregut and midgut to

retain food has also been reported for larvae of the freshwater goby Chaenogobius annularis and for Leiostomus xanthurus (Govoni 1980, Watanabe and Sawada 1985, both cited in Govoni et al. 1986).

Assuming that food is maintained at a constant density, and that feeding continues as a simple linear process throughout the day, daily food intake (ration) of first feeding larvae can be calculated as the product of GCT and gut content. Thus the daily ration for the artificial diet can be estimated to be between 0.398mg- 0.916mg (153-352% of body weight), compared to 0.667mg-1.021mg (256-392% of body weight) for larvae fed Artemia. Ingestion rates may vary with prey density (Werner and Blaxter 1980), temperature (Houde 1989) and larval age, so that comparisons between studies must be made with some caution. High ingestion rates have been reported for larvae of a range of species, and are generally restricted to early larvae fed at high prey densities. Among marine species, early larvae of Anchoa mitchilli, Archosargus rhomboides and Archirus lineatus (Houde and Schekter 1981), Paralichthys dentatus (Buckley and Dillman 1982) and Pseudopleuronectes americanus (Laurence 1977) have been reported to ingest up to 150-300% of body weight of live prey per day. Similarly early larvae of freshwater fish such as the bream Abramis brama may ingest over 250% of body weight per day (Marmulla and Rösch 1990). In contrast to the above figures, some studies have reported lower values of between 50-100% of body weight per day for first feeding larvae of Clupea harengus (Kiϕboe et al. 1987), Rutilus rutilis (Keckeis and Scheimer 1990), Perca fluviatilis (Marmulla and Rosch 1990) and Sparus aurata (Yufera et al. 1993).

In general, high ingestion rates are characteristic of early larvae, with recorded values declining during development. Thus Laurence (1977) found that ingestion by Pseudopleuronectes americanus larvae declines from 250-300% of body weight per day at first feeding to 25-50% of body weight per day during later larval development. Similarly, Scheimer et al. (1989, cited in Kamler 1992) found that ingestion of zooplankton by roach, Rutilus rutilis, larvae declined from 77% of body weight per day at first feeding to 40% per day by the time larvae had reached 10 mg body weight. In contrast, Kolkovski et al. (1993) report a logarithmic increase in weight-specific ingestion rate of rotifers by Sparus aurata larvae between 20 days and 32 days old.

Protease activity

Many studies have been made of the development of proteolytic enzyme activity in fish larvae (e.g. Alliot et al. 1980, Vu 1983, Buddington 1985, Cousin et al. 1987). However, there have been few studies relating enzyme activity to dietary factors, and particularly few investigations of the effects of quality and quantity of ingested diet

on enzyme responses in larvae (Rágyanski 1980, Baragi and Lovell 1986, Pedersen et al. 1987, Hjelmeland et al. 1989, Rösch and Segner 1990). This is perhaps not surprising in view of the problems associated with achieving ingestion of artificial diets by fish larvae at rates comparable to live feeds. In the present study, observations of larvae fed the dry artificial diet to the water surface suggest that this method of presentation results in poor acceptance and ingestion of the diet. However, the use of round-bottom flasks presented the diet in a way that encouraged ingestion by first-feeding larvae which was comparable (at least in dry weight terms) with that of larvae offered live food. Thus the results for enzyme activity in early larvae fed live food and artificial diet were made with similar amounts of each of food type in the gut.

The results for total protease and trypsin show a good correlation, at least for larvae of less than 12 mg, which indicates that the use of trypsin as an indictor of alkaline proteolytic activity in carp larvae is justified. The pancreas of first-feeding carp, and early larvae of most other fish species studied, is a diffuse organ along the gut (Smallwood and Smallwood 1931, Smallwood and Derrickson 1933, Stroband and Dabrowski 1981, Pedersen et al. 1987, Beccaria et al. 1991, Boulhic and Gabaudan 1992). It is likely that a large proportion of digestive enzyme secretion into the lumen of the gut may be across the intestine wall as well as through the pancreatic duct (Smallwood and Smallwood 1931, Dabrowski and Culver 1991, Boulhic and Gabaudan 1992). It has been suggested that pancreas of carp may not be fully functional until fish reach 4 cm in length (Smallwood and Derrickson 1933, Stroband and Dabrowski 1981). However, results of the present study indicate a strong increase in secretory capacity after metamorphosis (weight 10 - 14 mg, length 10 - 12 mm), which remains proportional to body weight thereafter. This increase corresponds well with the earliest weight (14 mg) at which mirror carp larvae can be weaned onto commercial trout fry diets without high mortality (Bryant and Matty 1981).

In the present study, modulation of digestive enzyme production in response to diet was observed in larvae before metamorphosis and the development of a fully functional pancreas. The results presented in Table 1a and Figure 9a show that at the onset of feeding, carp larvae have a very limited ability to respond to artificial diets with an increase in protease activity. In the first experiment there was no significant difference in protease activity between larvae fed the artificial diet and unfed larvae. Thus despite a full gut, there was no apparent stimulation of enzyme synthesis after ingestion of the artificial diet. In the second set of experiments, there was some evidence of a slight stimulation of trypsin activity in larvae fed the artificial diet, but this was questionable once the contribution of residual enzyme activity in the diet had

been allowed for (see Table 1). It should be noted, however, that in both cases these estimates are for enzyme activity within larvae at a fixed time. Given the rapid transit of food through the gut and the known inefficient retention of trypsin by cyprinid larvae (Hofer and Nasir Uddin 1986) it is possible that trypsin is in fact being secreted by larvae fed the artificial diet but with considerable losses in faeces.

In contrast, ingestion of Artemia by first feeding larvae produced an increase in total trypsin activity which can be attributed to both an exogenous dietary component, and an activation of trypsin secretion which is qualitatively similar to that previously recorded in marine fish larvae. The increase in trypsin activity over the 10h following initiation of feeding on Artemia nauplii corresponds to the de novo synthesis of trypsin recorded by Munilla-Moran and Stark (1989) in turbot larvae fed rotifers and by Pedersen et al. (1987) in herring larvae feeding on copepods. That idea that diet should make a significant contribution to total trypsin activity measured in fish larvae was first suggested by Dabrowski and Glogowski (1977a), and present results agree well with estimated values for exogenous protease activity in turbot larvae (Munilla-Moran et al. 1990).

Hjelmeland et al. (1988) found that there is a significant level of trypsin secretion in herring larvae fed polystyrene spheres, but that higher levels of trypsin were secreted in response to ingestion of zooplankton. They suggest that there may be a neural mechanism controlling pancreatic enzyme secretion in fish larvae, possibly mediated by stretch receptors in the gut. Over and above such mechanical stimulation, they argue that there might be a hormonally-mediated response to breakdown products from live prey which provide the main cues for trypsin secretion. In contrast, the results of Pedersen and Andersen (1992) suggest that neural cues predominate in the initiation of synthesis of trypsinogen by herring larvae. Hjelmeland et al. (1988) compare the digestive system of fish larvae to that of mammals, in which it is known that secretion of pancreatic enzymes is controlled by both neural and hormonal mechanisms (Rothman 1974). In mammals, in response to the influx of gastric chyme, hormones are secreted by the intestine (Preshaw 1974) which act to stimulate digestive enzyme secretion by the pancreas. Hjelmeland et al. (1988) suggest that in fish larvae a similar stimulatory role might be played by either low molecular weight molecules released through the cuticle of prey, or by products of autolysis of prey within the larval fish gut. In this model, the digestion of prey produces breakdown products which act to reinforce the hormonal signal stimulating pancreatic enzyme secretion. In addition to chemical cues, a neurally-mediated stimulation of trypsin production, acting through stretch receptors in the gut, might initiate or contribute to the feedback process. However, any control system must operate within the constraints set by the ontogenetic development of the digestive

organs. As outlined by Diamond (1986), ontogeny of digestive enzyme secretion in vertebrates may be 'hard-wired' with either central or local triggers initiating secretion at specific stages of development. Modulation of enzyme secretion in response to dietary factors can only operate within limits set by such fixed mechanisms.

In the interpretation of the present results at least two caveats should be noted. Firstly the use of whole fish homogenates in enzyme assays precludes both the localisation of enzyme activity and the separation of protease activity from precursors such as trypsinogen which are likely to be activated during sample preparation. Thus a diet may elicit activation of precursors and their secretion into the gut, which might be apparent as a reduction in whole tissue trypsin activity. Conversely, decreased trypsin release into the gut and accumulation of trypsinogen in the pancreas might yield a high trypsin activity in whole larval homogenates which could be wrongly interpreted as indicating as high level of trypsin production. Secondly, the high water content of Artemia nauplii (up to 90%, Watanabe et al. 1983a), means that the volume of gut contents and hence gut distention was very probably higher in larvae receiving the live food. Hence any potential signal from a mechanical cue via stretch receptors in the gut wall may have been greater than when an equivalent dry weight of artificial feed was ingested.

In Table 1b and Figure 9b it can be seen that carp larvae of 4 mg weight appear to have developed the ability to respond to the artificial diet with increased protease secretion. Although there was no significant difference in protease responses to the dietary treatments in larvae at 12-14 mg weight, results with postlarvae (Figure 6b) suggest that a similar response persists during later development. Using histochemical techniques Rösch and Segner (1990) found that Coregonus lavaretus larvae secreted more digestive enzymes in response to artificial diets than to live zooplankton, the response being attributed to the low digestibility of the artificial diet, with more efficient digestion of the zooplankton being achieved despite lower enzyme activity - an interpretation which appears equally applicable to the present study. There was also evidence of modification of digestive enzyme response to different dietary formulations. The use of histochemical techniques showed that there was an actual increase in enzyme activity within the gut lumen, suggesting that the elevated protease activity observed in the present study was similarly a consequence of enzyme synthesis and secretion rather than accumulation of stored precursors.

Hofer and Nasir Uddin (1984) found that trypsin activity was higher in roach larvae feeding on an artificial diet than in those fed zooplankton. As in the present study, despite the elevated trypsin levels in roach, larvae fed the artificial diet

exhibited poor growth and high mortality. Similar results are reported by Person-Le Ruyet (1993) for Dicentrarchus labrax larvae of over 4 mg weight. Hofer and Nasir Uddin (1984) also found that, in contrast to adult fish, larval roach did not reabsorb significant amounts of trypsin in the hind-gut. They argued that the losses resulting from continuous high enzyme secretion without recovery from the gut might contribute to the failure of the artificial diet to support larval growth and survival.

How a high trypsin secretion response to an artificial diet is initiated is not easily explained by the chemical cues or by stretch reception in the gut wall discussed earlier - as both these mechanisms would also be activated by ingestion of Artemia. It might be proposed that a feedback mechanism operates, whereby digestion of a meal (signalled by a high uptake of breakdown products) acts to reduce trypsin production. Thus feeding on either live or artificial diets would initiate trypsin secretion. However, the breakdown and release of the more readily digestible components of Artemia would rapidly signal a 'finished' meal and suppress trypsin secretion, whereas a less digestible artificial diet would not achieve the level of breakdown required to produce a cessation of enzyme production. Alternatively, it is possible that the observed low trypsin activity is a an artifact caused by high secretion of trypsin (which would be lost with faeces) and consequent depletion of trypsinogen in the pancreas.

Assimilation efficiency

Filatov (1972) has estimated energetic assimilation efficiencies of between 74% to 87% for Cyprinus carpio larvae of 1mg to 5mg dry weight (5 to 10 day old) feeding on zooplankton. In contrast Kamler (1992) calculated energy assimilation efficiencies between 20% to 40% for 10 to 15 day old Cyprinus carpio larvae feeding on zooplankton. Kamler (1992) also calculated that 5 to 10 day old carp larvae assimilated only 5 to 10% of energy from two artificial diets, rising to between 25% to 45% for larvae between 15 to 25 days old. The present study also indicates a higher efficiency of assimilation of energy from live food than the artificial diet by first feeding larvae, and agrees fairly well with Kamler's (1992) estimate of dietary assimilation for early larvae feeding on both types of food, given that the estimate for assimilation of the artificial diet used in the present study is likely to be high. Kolkovski et al. (1993) found that the dry weight matter efficiency of a microparticulate diet was only 4.8% for 20 day old Sparus aurata larvae, increasing to 55% for 30 day old larvae (in which acid protein digestion might be expected to have developed).

That early fish larvae should exhibit low assimilation efficiencies when feeding on zooplankton follows logically from the simple nature of the larval gut, but is not

immediately apparent from a review of the literature. Assimilation efficiencies can be dependent on prey density (Boelhert and Yoklavich 1984), being higher at low prey densities, and also may increase as the digestive system of larvae develops and ingestion rates decrease. Thus Houde (1989) has calculated average assimilation efficiencies for first feeding marine fish larvae of between 77% at 10^{o}C and 60% at 30^{o}C, based on ingestion, growth and metabolic rates reviewed from the literature. However, many of the studies from which these values were calculated use relatively low prey densities which can result in lower ingestion rates and high growth and assimilation efficiencies (Houde and Schekter 1981, Checkly 1984, Boelhert and Yoklavich 1984). Similarly Govoni et al. (1986) list assimilation efficiencies for a range of marine and freshwater species of between 45% to 99%, where in many cases prey densities were low (less than 1 prey item per ml). In comparison, Boelhert and Yoklavich (1984) calculated that the energetic assimilation efficiency of Clupea pallasi larvae was 68.2% when fed Artemia at a density of 1 l^{-1} but dropped to 38% when prey density was increased to 10^3 Artemia l^{-1}. Their results support the observation by Werner and Blaxter (1980) that high prey densities result in increased feeding rate and decreased assimilation efficiency in Clupea harengus larvae. Boelhert and Yoklavich (1984) liken the feeding strategy of early fish larvae to the 'superfluous feeding' identified in invertebrate zooplankton, which exhibit high ingestion rates and low assimilation efficiencies at high food densities (Beklemishev 1962). Griffiths (1975) suggested that fish larvae are 'number maximizers' as opposed to 'energy maximisers' in their feeding strategy, which is driven by the influence of prey density on ingestion rate. However, Boelhert and Yoklavich (1984) argue that it is impossible to distinguish between such strategies because although assimilation efficiency drops with feeding rate, total energy assimilation increases and hence growth is optimised. Thus the observed flexibility in feeding rate with prey density optimises energy assimilation in response to a patchy food resource in the natural environment.

A potential role for soluble protein in larval fish nutrition?

Although lipids are an important source of energy in the yolk of pre-feeding fish larvae, and may also contribute to the energy requirements of feeding larvae (see Chapter 2), protein appears to be the predominant source of energy in first-feeding larvae. Furthermore Dabrowski and Culver (1991) point out that the rate of protein synthesis in fish larvae may be up to an order of magnitude higher than in adults. Thus the rate of assimilation of dietary protein can be a critical rate-limiting step in maintenance of larval growth. Calculations based on measurement of ammonia-nitrogen excretion by first-feeding carp larvae indicate that metabolic oxygen consumption is largely attributable to oxidation of dietary protein (Kaushik and

Dabrowski 1983). However, extraction of protein from zooplankton prey by early larvae is relatively inefficient, as protein digestion appears to be largely mediated by pinocytotis and intracellular degradation of dietary macromolecular proteins in the hindgut epithelium (Watanabe 1984). Gardner (1984) has suggested that pinocytosis and intracellular digestion of proteins is a less efficient mechanism of digestion than hydrolysis and active uptake across the gut wall. However, zooplankton contain high levels of soluble proteins which may be readily available for this route of uptake. According to Berges et al. (1990) soluble protein can represent 15% of dry weight of Artemia nauplii. 11-14% of dry weight of rotifers may be made up of soluble protein, of which 60% may be digested to relatively small peptides after 2 h in vitro post-mortem autolysis (Hjelmeland et al. 1993). In the same study, only 5% of soluble protein was available as free amino acids after 2 h autolysis. Although Fyhn (1989) has suggested an important role for free amino acids in the nutrition of first-feeding fish larvae, it seems likely that the soluble protein fraction of prey may predominate in larval nutrition.

The low assimilation rate which accompanies high feeding rates in early larvae might be largely achieved by uptake of nutrients from the pool of soluble components within the prey following slight physical damage during ingestion and the action of both prey autolysis and endogenous digestive enzymes. Based on a mean daily ingestion rate 844μg of Artemia (Table 4), with a soluble protein content of 15% of dry weight (Berges et al. 1990) it can be calculated that soluble protein intake contributes 2.8J (16%) of daily energy intake by first feeding carp larvae (Winberg 1971). From Table 4 it can be seen that the combination of energy used for metabolism and growth by first feeding larvae is 3.4J, suggesting that potentially a substantial part of, if not all, the total protein requirement of larvae could be met by the soluble protein component of live food. First feeding carp larvae excrete 4.2 μg N.mg^{-1} wet weight per day, primarily as ammonia (Kaushik and Dabrowski 1983, Kamler 1992). Converting by a factor of 5.1 (Jobling 1983), excreted nitrogen represents oxidation of 42 μg of dietary protein for a fish of 2mg body weight. That amount of protein would represent 35% of the pool of solubles contained in ingested Artemia, leaving 65% (79μg) potentially available for protein deposition. Given that live prey such as Artemia will also supply readily assimilable energy in the form of carbohydrates and fatty acids (Govoni et al. 1986), there appears to be little requirement for digestion of insoluble dietary protein by early fish larvae when fed live prey at high densities. As the efficiency of enzymatic breakdown of food protein in the larval gut is dependent on the product of enzyme activity and gut transit time ('proteolytic action' or PA, Hofer and Nasir Uddin 1985), the increase in gut clearance time at lower food densities presumably results in the more efficient

digestion of protein required to support metabolism and growth.

A strategy of a high ingestion rate coupled with low digestive enzyme activity (low PA) is ineffective when larvae are offered a high density of an attractive but relatively stable artificial diet. Artificial diet particles may not contain high levels of soluble components as a result of processing, or may already have suffered extensive leaching before ingestion. Diet production processes can alter the digestibility of food proteins, so that pinocytotic protein uptake is greatly impaired. For instance, Bengston et al. (1993) have found that alginate encapsulation of Artemia results in poor uptake of protein in the posterior intestine epithelial cells of Menidia beryllina larvae. In comparison, rectal and posterior intestinal epithelial cells of larvae fed live Artemia were found to contain lipo-proteins originating from the prey. Damage to Artemia during encapsulation may also have resulted in leaching of soluble components from diet particles. In the present case, the artificial diet formulation contained 15% soluble protein, but this was lost by leaching within 1h of hydration (Dr. J. R. Stark, pers comm). Losses from non-encapsulated microparticulate diets can be substantially higher - representing up to 50% of protein and dry matter less than one min after hydration (Kamler 1992).

As fish larvae develop, there is an increase in weight-specific protease activity coupled with increasing gut clearance time and decreasing daily ration. The consequent change to a strategy of highly efficient assimilation of relatively smaller quantities of live prey means that older larvae are also able to extract sufficient energy from relatively indigestible artificial diets to survive and grow. This is true for larvae of stomachless species such as carp, as well as for cultured marine species such as Dicentrarchus labrax, for which effective weaning becomes feasible during the period before development of acid digestion when increased secretion of pancreatic enzymes in response to artificial diets has developed (Person Le Ruyet et al. 1993). In the present study, there was an increase in PA for larvae fed the artificial diet from between 1.8 Unit hours at 2mg body weight to 36 Unit hours at 4mg body weight. Even after 10 days, larvae fed the artificial diet had only just reached a size where an elevated enzyme response to the artificial diet and increase in gut clearance time might be expected. Thus assimilation of the artificial diet is so low at first feeding that some larvae are unable to achieve energy intake beyond the 'break even' point - and effectively starve - while those that can assimilate enough energy to support some growth are 'held back' in a stage of development which is poorly adapted to feeding on an artificial diet.

Feeding regimes and the use of artificial diets

In the present study, the mean rate of ingestion of the spray-dried diet

by first-feeding carp larvae in flasks was 75% of that for live food, although no statistical difference was found. In contrast, the very low amount of artificial diet ingested in the first larval rearing experiment is more representative of typical of culture systems. However, even when fed in flasks, low ingestion of the diet may have contributed to mortality and to poor growth in survivors. Low ingestion rates of dry food have been identified as an important limiting factor in the successful application of artificial diets. Using ^{14}C labelled diets, Kolkovski et al. (1993) found that 22 day old (0.5mg dry wt) Sparus aurata larvae ingested approximately 10 times more rotifers h^{-1} than a microparticulate diet. Such low ingestion rates coupled with a low assimilation efficiency (6% for 20 day old larvae) were identified as the causes of low growth of Sparus aurata fed the artificial diet. Similarly, Weinhart and Rosch (1991) have identified low ingestion rates as one of the underlying reasons for low growth of Coregonus lavartus larvae fed dry food. In the present study, larvae which exhibited low ingestion of the artificial diet will have been greatly disadvantaged by the combination of poor diet digestibility and low throughput of food.

There is clearly scope for improvement in methods of presentation of artificial diets to fish larvae - particularly in terms of frequency and density of feeding. Optimisation of food density, meal duration and intervals between meals might improve efficiency of assimilation of the diet in carp larvae - and may improve results with smaller marine larvae. In commercial culture systems diets must be added to the surface of rearing tanks and should be prehydrated to promote rapid dispersion of particles throughout the water column. The shape of rearing tanks, and water circulation in the tanks must maximise suspension and movement of diet particles - while allowing easy removal of uneaten material.

A high density of particles fed periodically (at intervals of more than the observed GCT under continuous feeding) might encourage high ingestion followed by extended gut retention in the absence of further food. Charlon and Bergot's (1984) successful culture of carp larvae on an artificial diet used frequent discrete addition of food to the rearing tanks, which were specially designed for use with artificial diets. The use of frequent (hourly) feeding of artificial diets has been used successfully in the rearing of carp larvae in the system of open tubes with mesh bottoms described in the present study, though again with relatively poor growth rates (Monroy 1992). Also, a feeding protocol which encourages rapid ingestion of diet particles will also reduce exposure of the diet to leaching in the culture water, although loss of the most soluble dietary components takes place within minutes, if not seconds, of hydration.

Use of carp larvae as a model in the testing of larval fish diets

The larvae of some freshwater species, such as carp, are better able to adapt to

artificial diets than are larvae of most marine species (see review Chapter 1). However, in the present study, the failure of the Frippak diet to support high survival and growth in carp larvae is attributable to the low level of digestive proteases produced by first-feeding larvae, coupled with a lack of readily assimilated nutrients in the diet. In this respect, the problems faced in producing artificial diets suitable for first-feeding carp are very similar to those experienced with marine larvae. The present study has demonstrated that carp larvae can provide a valid, relatively robust and readily available model for the testing of novel forms of artificial diet. The ready acceptance of artificial diets by carp larvae allows the behaviour of diet particles in the larval gut to be followed fairly easily, without the problems of low ingestion rates associated with early marine larvae. Once microencapsulated diets have been developed which can deliver effective levels of soluble or readily digestible nutrients to early carp larvae, the application of such technology to marine species might be more easily undertaken.

Prospects for diet development

From the results of the present study it is clear that the spray-dried form of microencapsulated diet, in common with other forms of microencapsulated particle such as cross-linked protein microcapsules (Walford and Lam 1991) and alginate wall microcapsules (Bengston et al. 1993), is unsuitable as a food for first-feeding fish larvae due to problems of leaching of soluble components, indigestibility of insoluble protein and the physical stability of the diet during the rapid transit of particles through the gut. Furthermore, the resulting poor assimilation of nutrients from artificial diets tends to be exacerbated by low ingestion rates. The feeding of early fish larvae with an artificial diet presents a paradox; the need for diet particles sufficiently stable to survive drying and handling and with low leach properties must be balanced against the inability of the larvae to physically or enzymatically break down such particles. The process of microencapsulation increases particle stability and reduces leaching of nutrients to a certain extent but at the cost of greatly reduced digestibility. This perhaps explains the better results experienced with microbound diets (Table 2, Chapter 1) which although prone to extremely rapid leaching (Kamler 1992) are more porous and less physically stable than microcapsules - resulting a greater surface area for digestion, particularly of components such as lipids which may remain inaccessible within a microencapsulated particle. However, as discussed in the previous section, the loss of leachable components from either type of particle will reduce their suitability for fish larvae.

One approach to improvement of the assimilation of dietary protein is to include digestive enzymes in the artificial diet formulation. Initial attempts by Dabrowski

and Glogowski (1977b) and Dabrowska et al. (1979) to supplement carp larval diets with pancreatin met with limited success. This may have been due to the advanced stage of development of the fish used which, as the present study has shown, are able to increase protease activity in response to an artificial diet. More recently, Kolkovski et al. (1993) have experimented with the addition of porcine pancreatin to a gelatin microbound diet for Sparus aurata larvae. They found that a 0.05% w/w inclusion of pancreatin improved dry matter assimilation efficiency from 4.8% to 6.1% in 20 day old larvae, resulting in significantly better growth (SGR = 0.078) than in larvae fed the diet without pancreatin (SGR = 0.034). However, larvae fed rotifers showed significantly better survival and growth than either of the artificial diet treatments - which was attributed to higher rates of ingestion and assimilation of the live food.

Maugle et al. (1983) made cross-linked protein microencapsules containing amylase and protease, which were included in penaeid shrimp ongrow diets. The enzymes were found to survive the encapsulation procedure and produced improvements in dietary assimilation and shrimp growth. The preliminary observations made in the present study demonstrate that there is potential for the development of a protein-wall microcapsule containing pancreatin which would provide the predigested nutrients required by early larvae within a particle which is physically unstable in the larval gut. It was particularly encouraging that microcapsules which had been freeze-dried retained sufficient enzyme activity to autolyse within the gut - suggesting that long term storage might be possible.

An obvious disadvantage to the use of diets containing digestive enzymes is that in a rearing system where a large proportion of diet remains uneaten, as is the case with fish larvae, autolysis of settled particles will result in loading of the culture water with nutrients and a consequent loss of water quality. Such problems might be avoided by tank design and water exchange management - and might be no worse than in the case of a microbound diet subject to very high leach loss.

An alternative approach follows from the observation by Walford et al. (1991) that ingestion of rotifers together with protein-wall microcapsules enhanced digestion of the diet particles in Lates calcarifer larvae. It has been shown in the present study that microcapsules containing relatively high levels of pancreatin can be co-fed with a standard encapsulated diet in order to aid digestion within the larval gut (Plate 5b). This approach offers the advantage of improved digestion of the artificial diet, which is not brought into close contact the pancreatin until ingested - hence aiding water quality maintenance.

The results of the present chapter clarify the need for a new type of artificial diet to meet requirements set by the feeding strategy and digestive capacity of early fish larvae. The inclusion of digestive enzymes remains an attractive prospect,

although much further work remains to be done. It should be possible to avoid the use of cross-linked microcapsules, which are difficult to prepare on a large scale. Spray-dried microcapsules can be easily and relatively cheaply mass-produced, and are the most suitable alternative at present. The observation of measurable levels of trypsin in the diets used in the present study suggests that addition of digestive enzymes to this type of diet is technically feasible and manipulation of spray-drying conditions should allow digestive enzyme additives to survive processing.

Table 1(a); Summary of data used to calculate endogenous trypsin activity in 2mg carp larvae (24H after start of feeding). Calculated ranges are based on mean values for diet enzyme activity and 95% confidence intervals for gut contents and larval trypsin activities.

Diet	Total trypsin activity +/- s.d. (mU/larva)	Gut contents, (mg dry weight)	Exogenous trypsin activity		Net endogenous trypsin activity mU/larva
			mU/larva	%	
Artemia	3.29 - 3.74	0.046 - 0.071	0.58 - 0.90	15.5 - 24.1	2.39 - 3.16
Frippak CD	0.88 - 1.47	0.028-0.064	0.07 - 0.17	4.9 - 18.7	0.71 - 1.40
Unfed	0.30 - 0.74				0.30 -0.74

Table 1(b); Summary of data used to calculate endogenous trypsin activity in 4mg carp larvae (72 - 96H after start of feeding). Calculated ranges are based on standard deviation ranges for diet trypsin activities and 95% confidence intervals for gut contents and larval trypsin activities.

Diet	Total trypsin activity (mU/larva)	Gut contents, (mg dry weight)	Exogenous trypsin activity		Net endogenous trypsin activity mU/larva
			mU/larva	%	
Artemia	2.06 - 5.54	0.064 - 0.138	0.81 - 1.75	14.6 - 85.1	0.31 - 4.73
Frippak CD	7.07 - 11.28	0.079 - 0.149	0.20 - 0.38	1.8 - 5.4	6.89 - 11.08
Unfed	2.65 - 4.00				2.65 -4.00

1. Calculations use diet trypsin activities of;
   Artemia 12.7 mU / mg dry wt.
   Frippak CD 2.57 mU / mg dry wt.

2. For trypsin assays n= 6 to 9 (10 to 20 larvae/sample)

Table 2. Calculation of assimilation efficiency for three day old carp larvae fed Frippak CD diet or *Artemia*

	Artificial diet.		*Artemia*	
	Mass	Energy (J)	Mass	Energy (J)
Larval wet weight (mg)	1.9		1.9	
Larval dry weight (ug)	263.0		263.0	
GET (min)	100.0		100.0	
Gut content (ug)	45.6		58.6	
Daily ration (ug)	656.6	15.10	843.8	17.72
Growth (%)	13.0		32.0	
Growth (ug)	34.2	0.99	84.2	2.44
Metabolism		0.95		0.95
Assimilation efficiency for energy (%)		12.9		19.1

Notes;

Assimilation efficiency calculated as Ka = (Growth + metabolism) / Daily ration

Oxygen consumption of fed larvae taken as 1.5 mg/g fresh wt/h (Kaushik & Dabrowski 1983)

Oxycaloric equivalent taken as 450kJ/mol (Weiser et al 1988)

Caloric equivalent of larval tissue taken as 29kJ/g dry wt (Weiser et al 1988).

Energy content of artificial diet calculated as 23 J/mg dry wt from proximate composition using factors of 17.14 J/mg (carbohydrate) 23.42 J/mg (protein) and 39.31 J/mg (lipid) (Winberg 1971) . *Artemia* energy content taken as 21 j/mg dry weight (Chapter 4).

Ingestion rates as calculated in text, mean growth rates calculated for 10 day period from start of feeding (see Tables 3 and 4).

Table 3. Some published specific growth rates (SGR) for Cyprinus carpio larvae reared using live zooplankton food.

Source	Diet	Temp (°C)	Initial size/age	Duration (Days)	S.G.R. (%)
Hamada et al. (1980)*	Limnodrilus	25	2mg	10	36.4
Kainz & Gollman (1980)**	Zooplankton (mixed)	21	1mg	(to 31mg)	24.5
Kainz & Gollman (1980)**	Zooplankton (mixed)	21	31mg	(to 400mg)	14.2
Bryant & Matty (1980)	Artemia	24	1.5mg	5	47-51
Bryant & Matty (1980)	Artemia	24	Day 6	5	31
Dabrowski (1984)	Zooplankton (unspecified)	25	1.5mg	14	25.5
Szlamińska (1987)**	Zooplankton (mixed)	24	1.5mg	(to 8.5mg)	24.8
Szlamińska (1987)**	Zooplankton (mixed)	24	8.5mg	(to 18.5mg)	11.2
Kamler et al. (1992)	Zooplankton (unspecified)	26	Day 3	14	36.6
Present study	Artemia	24	1.5mg	10	31.4
Present study	Artemia	24	40mg	10	27
Chapter 2 (this volume)	Rotifers (enriched)	24	1.5	9	35
Chapter 2 (this volume)	Rotifers (unenriched)	24	1.5	9	31

* cited in Bryant and Matty (1981)

** cited in Kamler (1992)

Table 4. Some published values for specific growth rate (SGR) and survival of *Cyprinus carpio* larvae and post-larvae reared using artificial diets.

Source	Diet	Temp (°C)	Initial size/age	Duration (days)	Survival (%)	S.G.R. (%)
Appelbaum (1977)*	Yeast based	22	1.2mg	(to 43mg)	n.d	19.8
Bryant & Matty (1981)	Trout fry	24	2.4mg	18	2	5.1
Bryant & Matty (1981)	Trout fry	24	36.0mg	10	80	14.8
Dabrowski (1984)	Yeast/ liver	25	1.5mg	14	n.d	7.2-15.8
Dabrowski (1984)	Yeast/ liver	25	18.3mg	6	n.d	11.0-11.4
Charlon & Bergot (1984)	Yeast/ liver	23	Day 3	28	82-93	18.2-20.3
Charlon *et al.* (1986)	Yeast/ liver	20-24	Day 3	7	87	23.0
Charlon *et al.* (1986)	Yeast/ liver + trout fry	20-24	14 days	7	n.d	8.0
Szlaminska & Pryzybyl (1986)	Yeast based	24	1.6mg	(to 3.1mg)		9.6
Bergot *et al.* (1989)*	Yeast based	24	7.5mg	(to 46mg)	n.d	26.1
Bergot *et al.* (1989)*	Yeast based	24	46mg	(to 200mg)	n.d	22.3
Kamler *et al.* (1993)	n.d	26	Day 3	14	n.d	14.6-24.2
Present study	Frippak	24	1.5mg	10	16	13.3
Present study	Frippak	24	40mg	10	96	16.0

* cited in Kamler (1992)

n.d = no data

Figure 1. Schematic design of recirculating system used in larval experiments.

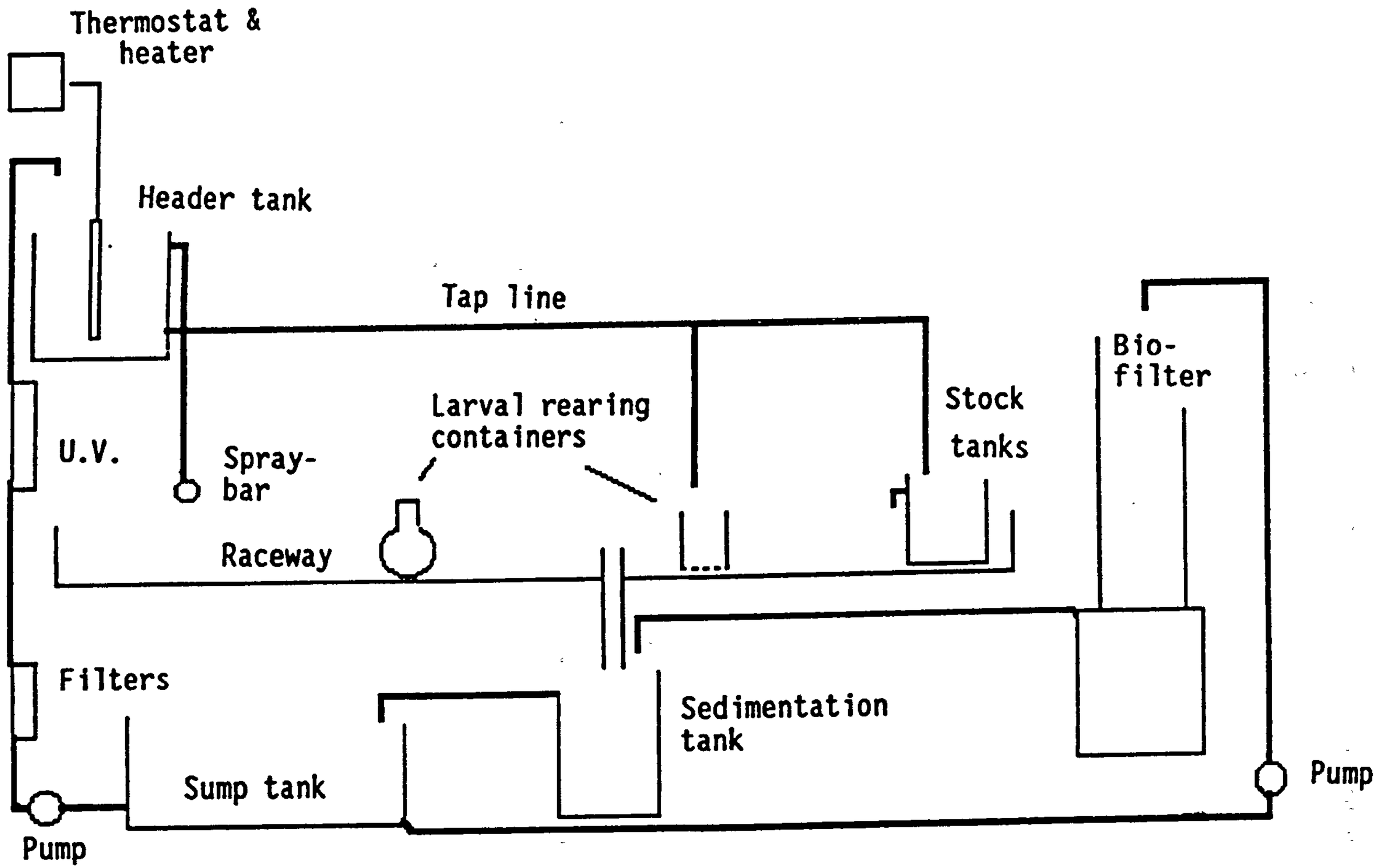


Figure 2a. Length (mm) of postlarval mirror carp fed Artemia or artificial diet. Data are pooled from three replicates, values are means ± s.d. , n = 45. Lines overlaying data points are fitted linear regressions of length against time for data pooled from three replicates for each treatment, as described below.

Regression of length (mm) against time (days)

	Artemia			Artificial		
	Fit	C.I.	t	Fit	C.I.	t
Intercept	13.65	0.40	66.66	14.96	0.43	69.02
Slope	1.52	0.06	49.09	0.64	0.06	19.52

Figure 2b. Ln weight (mg) of postlarval mirror carp fed Artemia or artificial diet. Values are means, ± s.d., calculated from weighing groups of 15 fish in each of three replicates. Lines overlaying data points are fitted linear regressions of ln weight against time for each treatment, as described below the figure.

Regression of ln weight (mg) against time (days)

	Artemia			Artificial		
	Fit	C.I.	t	Fit	C.I.	t
Intercept	3.81	0.08	103.6	3.79	0.16	52.51
Slope	0.27	0.02	36.50	0.16	0.03	11.09

Figure 2a.

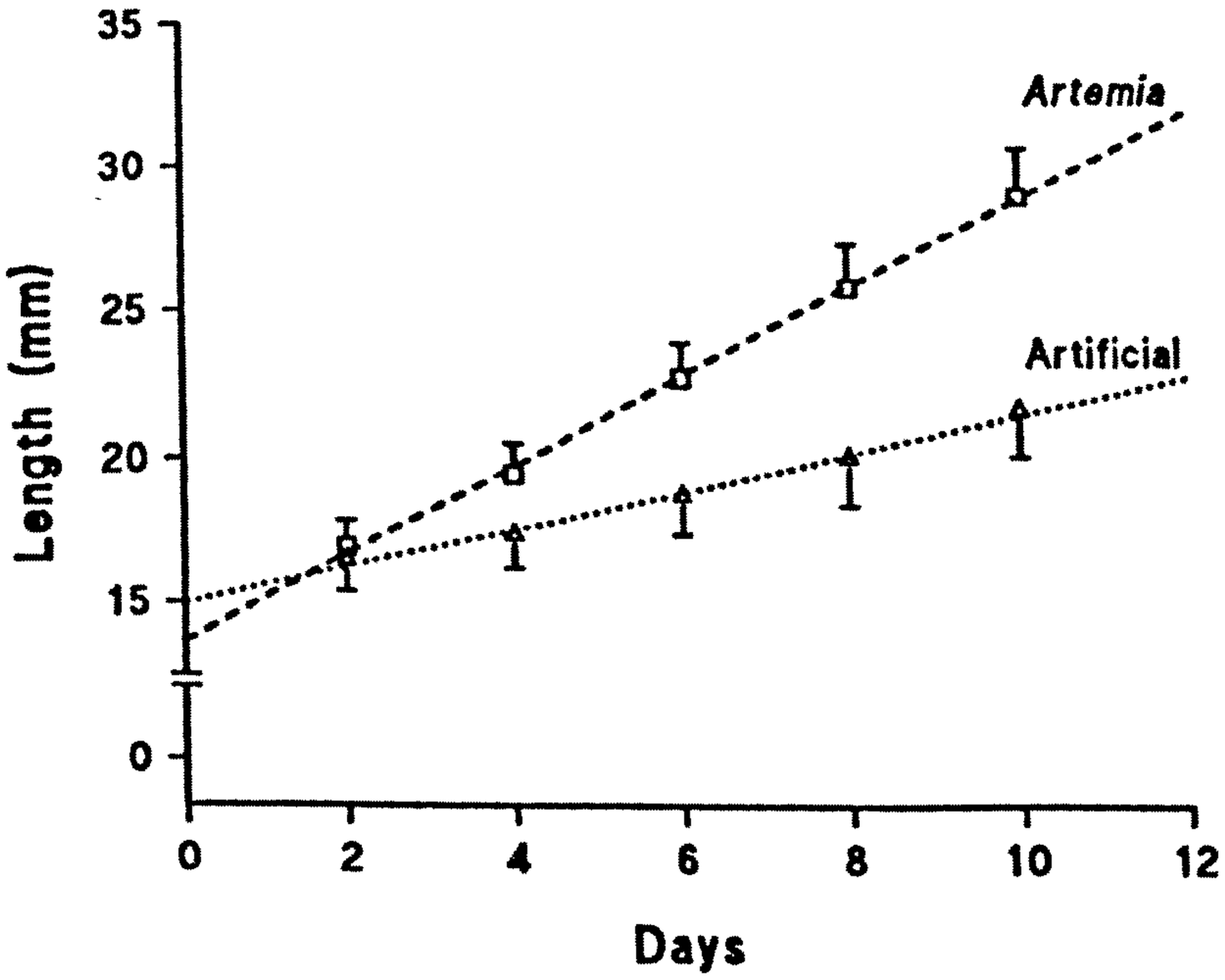


Figure 2b.

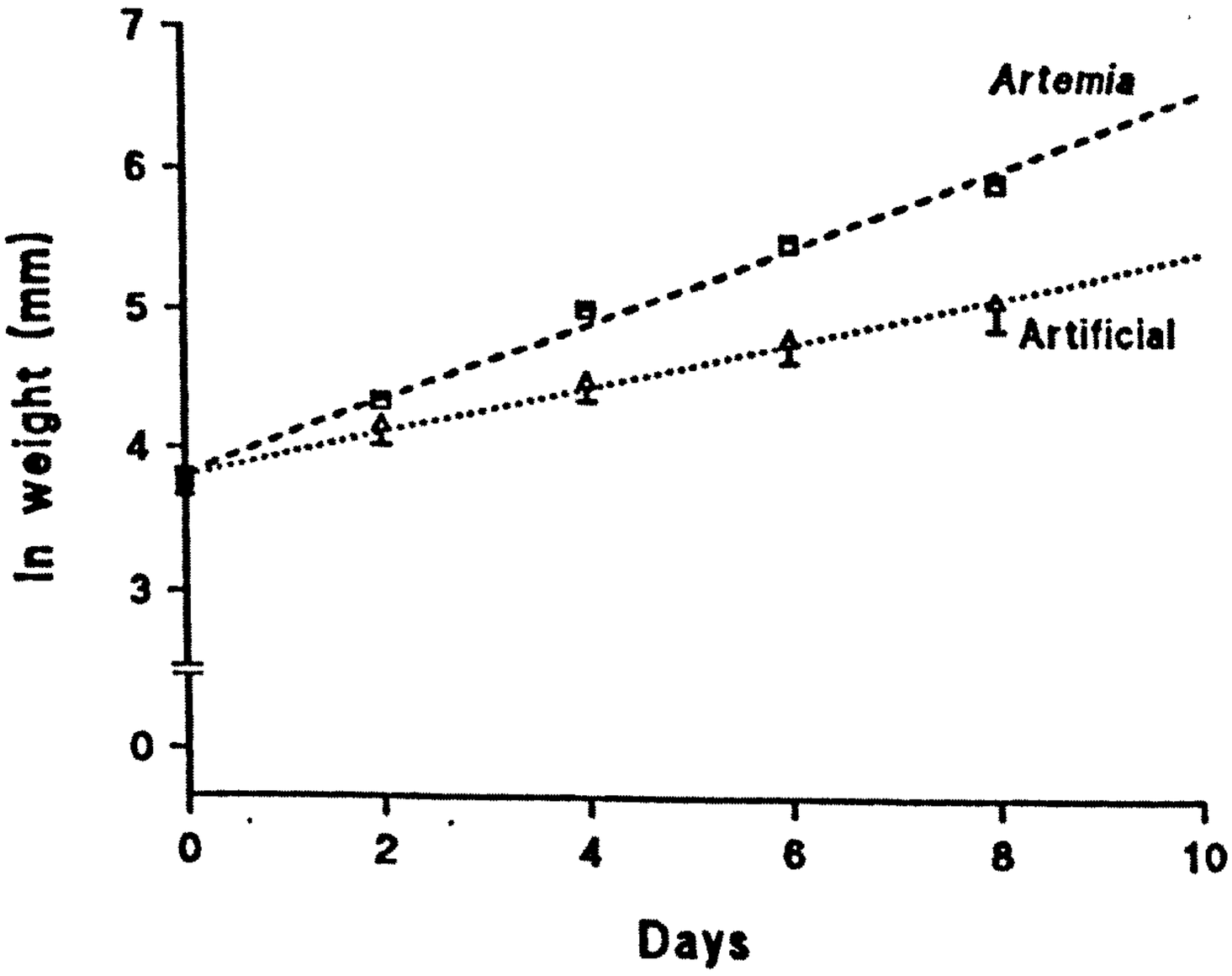


Figure 3a. Numbers of surviving carp larvae in treatments reared reared on Artemia nauplii or Frippak CD3 from first feeding (data pooled from three replicate containers).

Figure 3b. Length (mm) of carp larvae reared on Artemia or Frippak CD3 from first feeding (data pooled from three replicate containers, values are means ± s.d.).

Figure 3a.

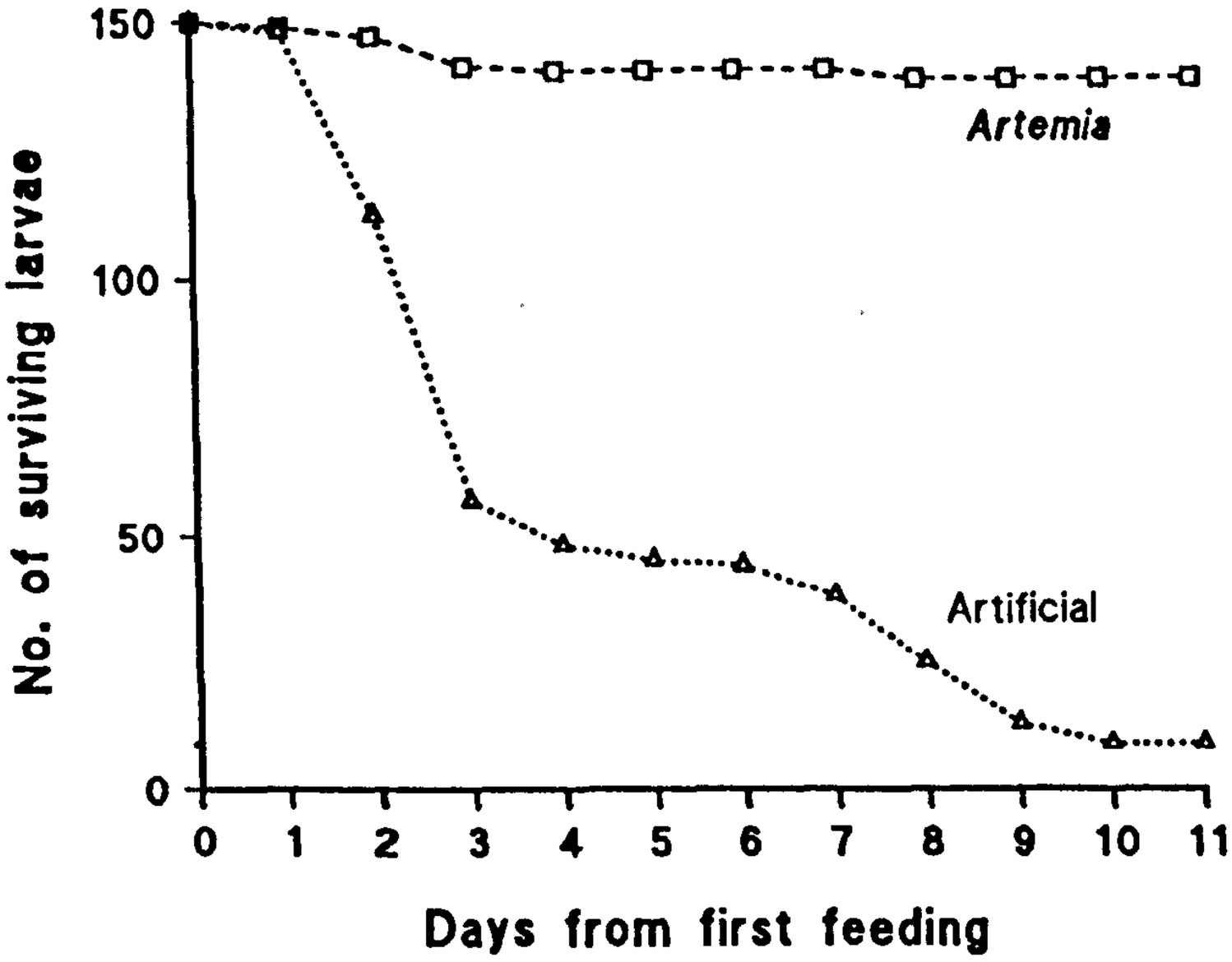


Figure 3b.

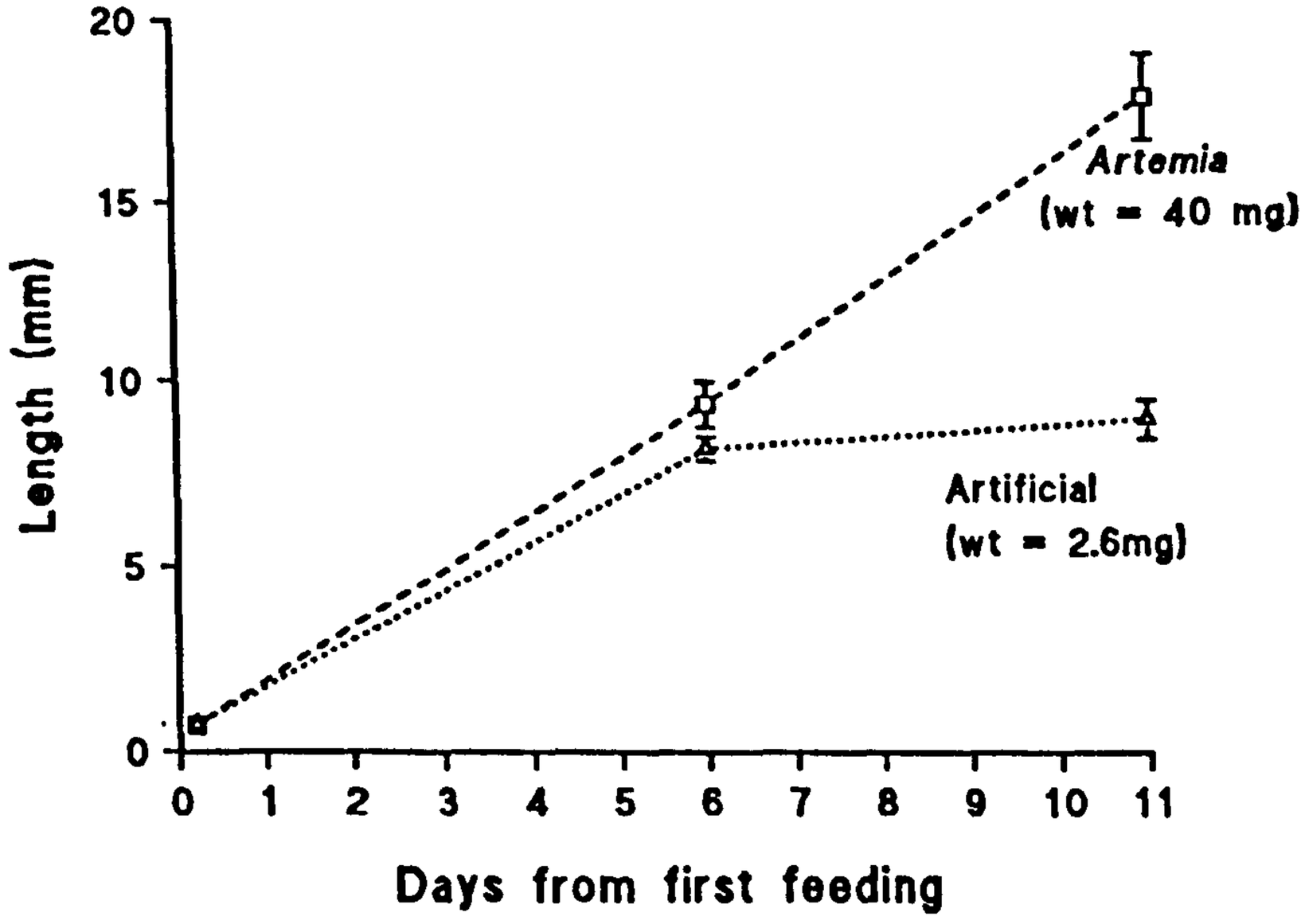
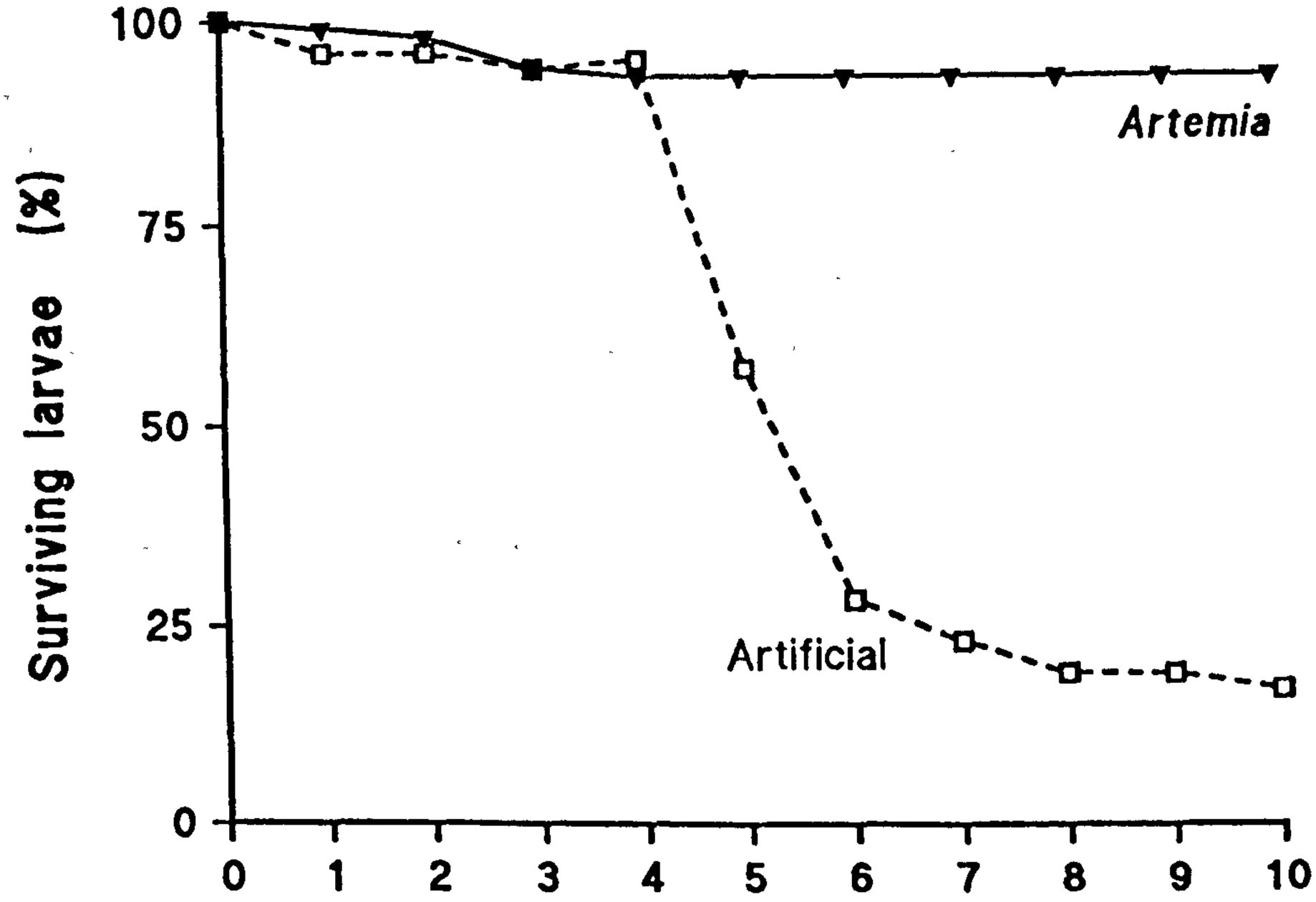


Figure 4. Survival (%) of carp larvae fed Artemia, or Frippak CD3 artificial diet in round-bottom flasks. Values are means from 3 replicate flasks for each treatment.

Figure 4.



	Artemia	Artificial
Weight at day 10 (mg)	34.5 +/- 8.3	5.7 +/- 2.4
Length at day 10 (mm)	15.3 +/- 2.5	6.2 +/- 3.2

Figure 5. Feeding responses by carp larvae offered Frippak CD3 artificial diet, distributed on water surface in open containers, during the first 6 days from first-feeding. Responses were recorded as;

1. Encounter: Food particle investigated but not taken into mouth.
2. Accept: Food particle taken into mouth and swallowed.
3. Reject: Food particle taken into mouth but rejected.

100 observations recorded for each day.

Figure 5

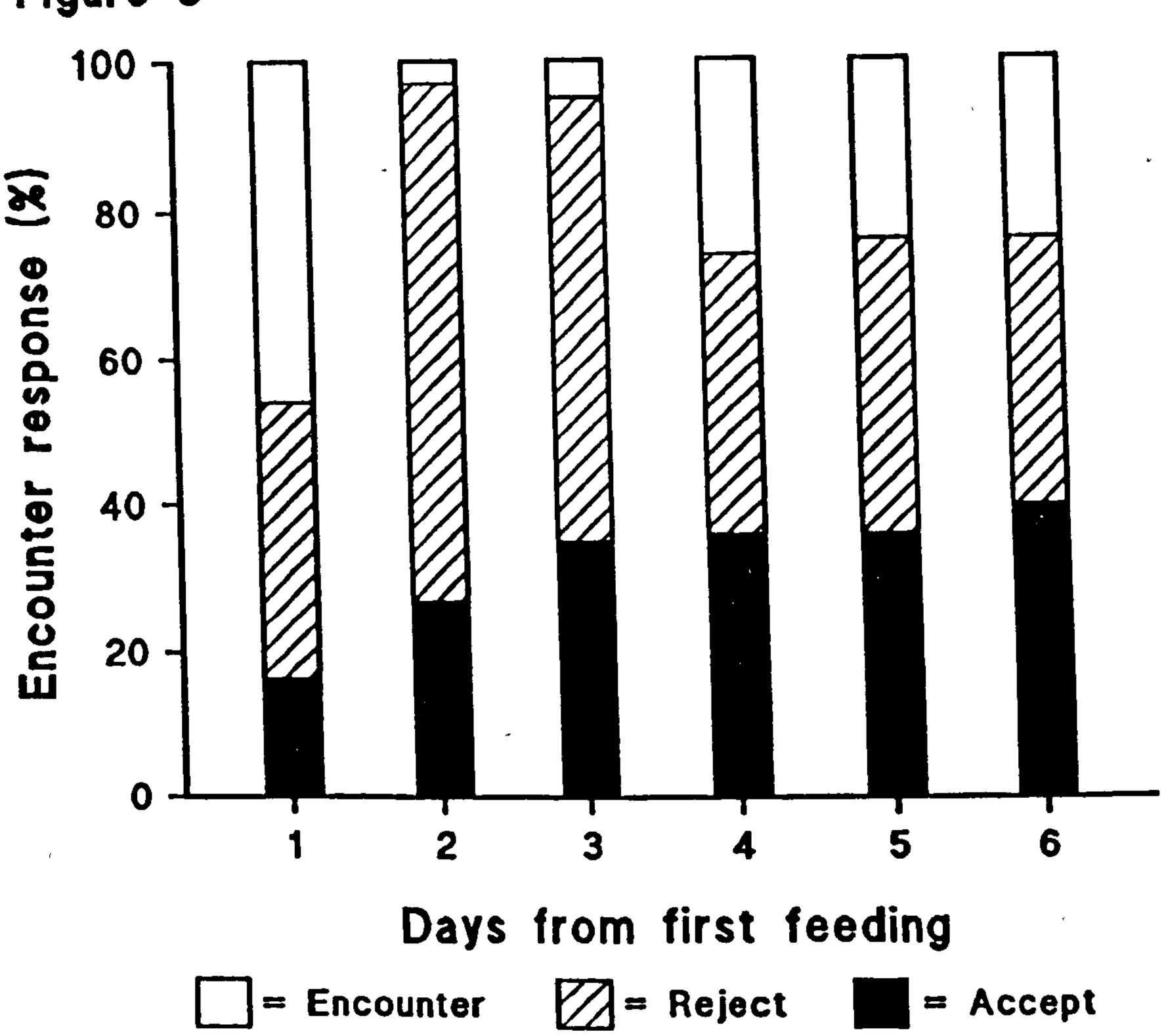

Encounter response (%)
100
80
60
40
20
0
1
2
3
4
5
6
Days from first feeding
= Encounter
= Reject
= Accept

Figure 6a. Position of the boundary between marked and unmarked Artemia in the gut of first feeding carp larvae, expressed as a percentage of gut length, plotted against time (min) from changeover. Each data point represents 5 obervations, error bars are standard deviations. Fitted linear regression lines overlay data, with equations inset.

Figure 6b. Position of the boundary between marked and unmarked artifical diet in the gut of first feeding carp larvae, expressed as a percentage of gut length, plotted against time (min) from changeover. Each data point represents 5 obervations, error bars are standard deviations. Fitted linear regression lines overlay data, with equations inset.

Figure 6a.

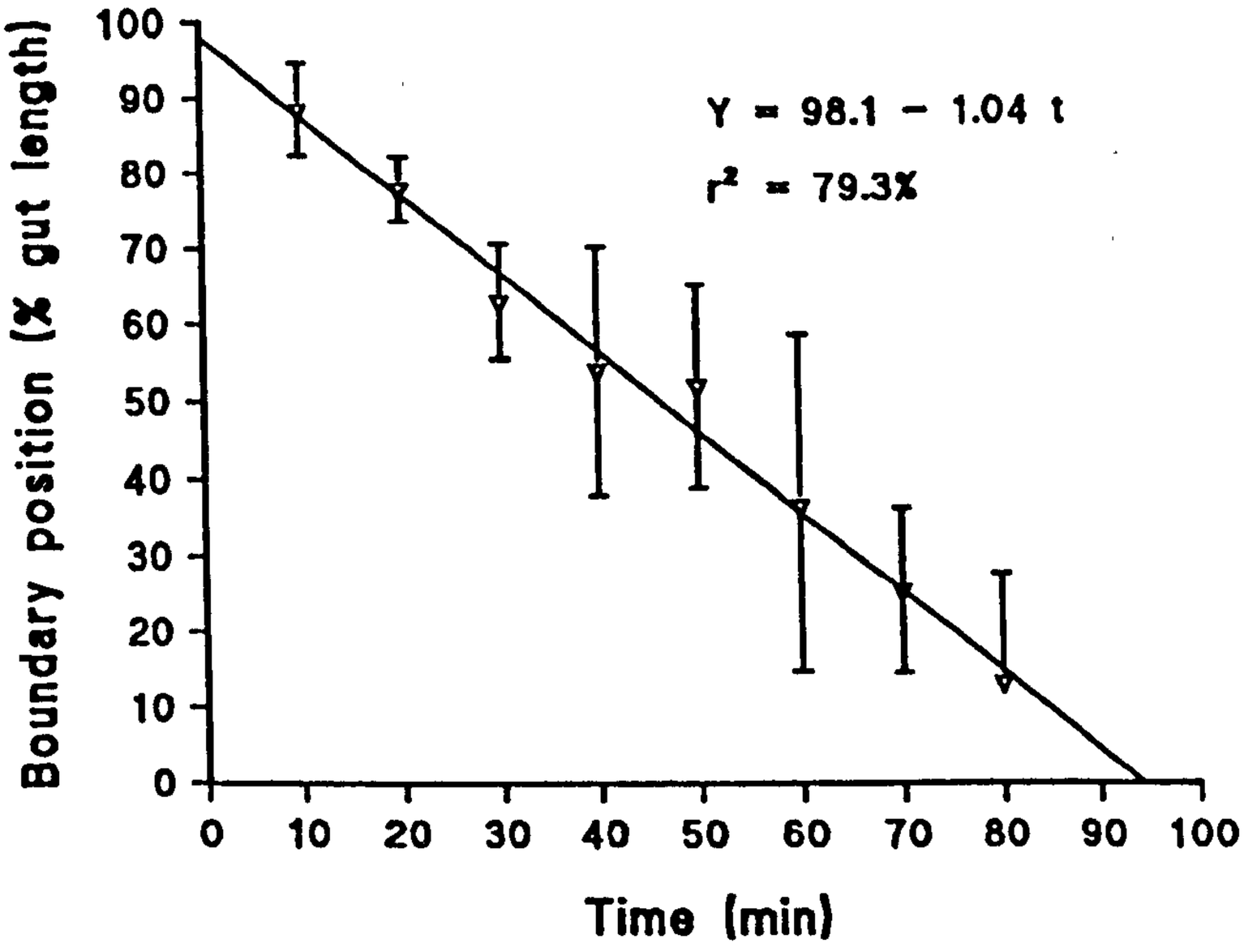


Figure 6b.

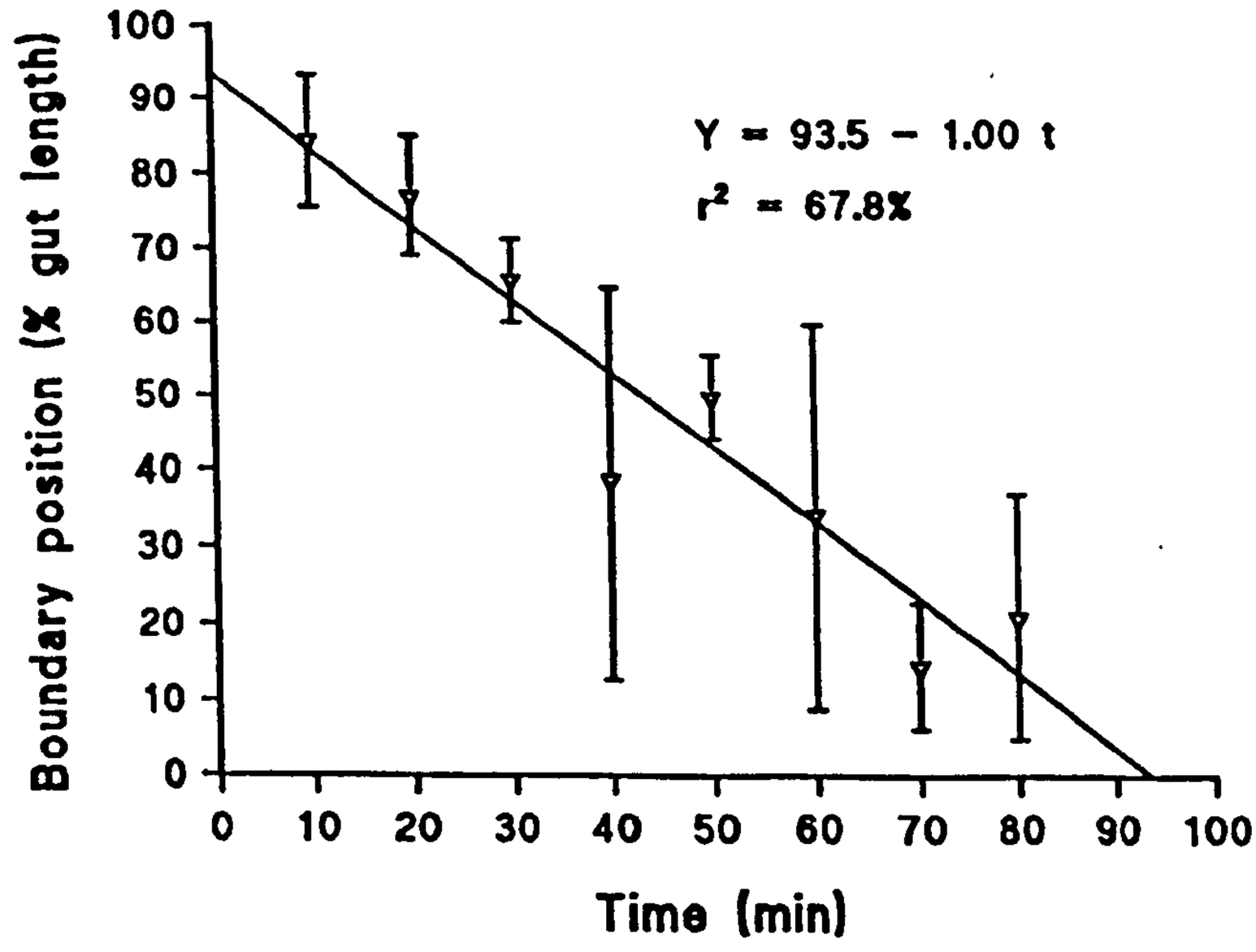


Figure 7a. Trypsin activity ($x10^{-3}$ units $larva^{-1}$) in carp larvae reared on Artemia nauplii to five weight ranges and then transferred to the following dietary regimes for 24h: starved, fed Artemia nauplii, fed Frippak CD3 artificial diet. Values are means ± s.d. (n = 3 to 6) for each point. Within each size range, treatments bearing different superscripts produced significantly different levels of trypsin activity ($p<0.05$).

Figure 7b. Trypsin activity (units $animal^{-1}$) in carp postlarvae fed Artemia nauplii or artificial diet. Lines overlaying data are fitted linear regressions of trypsin activity against weight for each treatment, as described below.

Regression of trypsin activity against weight (mg)

	Artemia			Artificial		
	Fit	C.I.	t	Fit	C.I.	t
Intercept	-0.059	0.249	-0.61	-0.060	0.198	-0.77
Slope	0.010	0.004	6.37	0.017	0.004	12.17
	r = 0.944			r = 0.984		

Figure 7a.

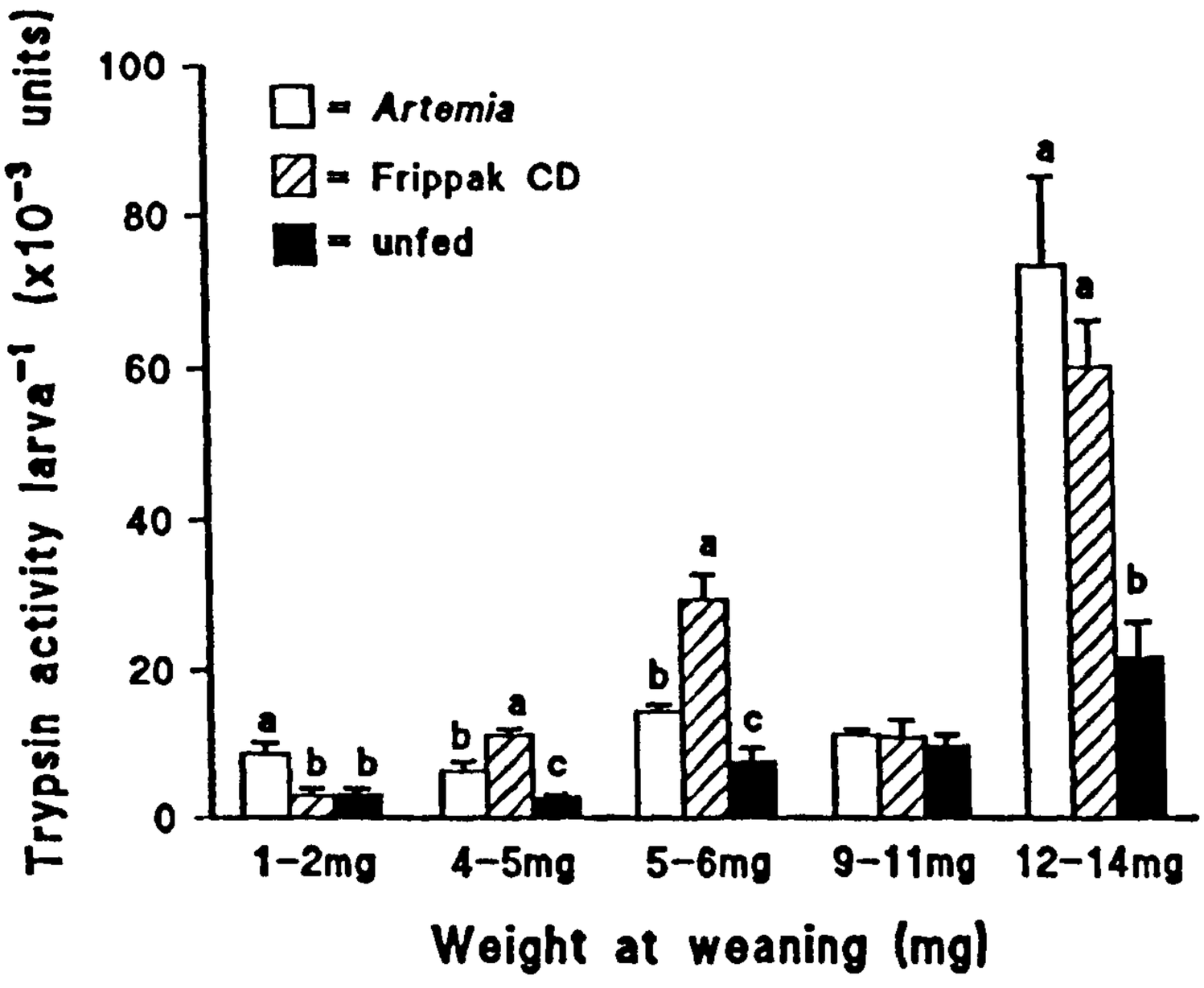


Figure 7b.

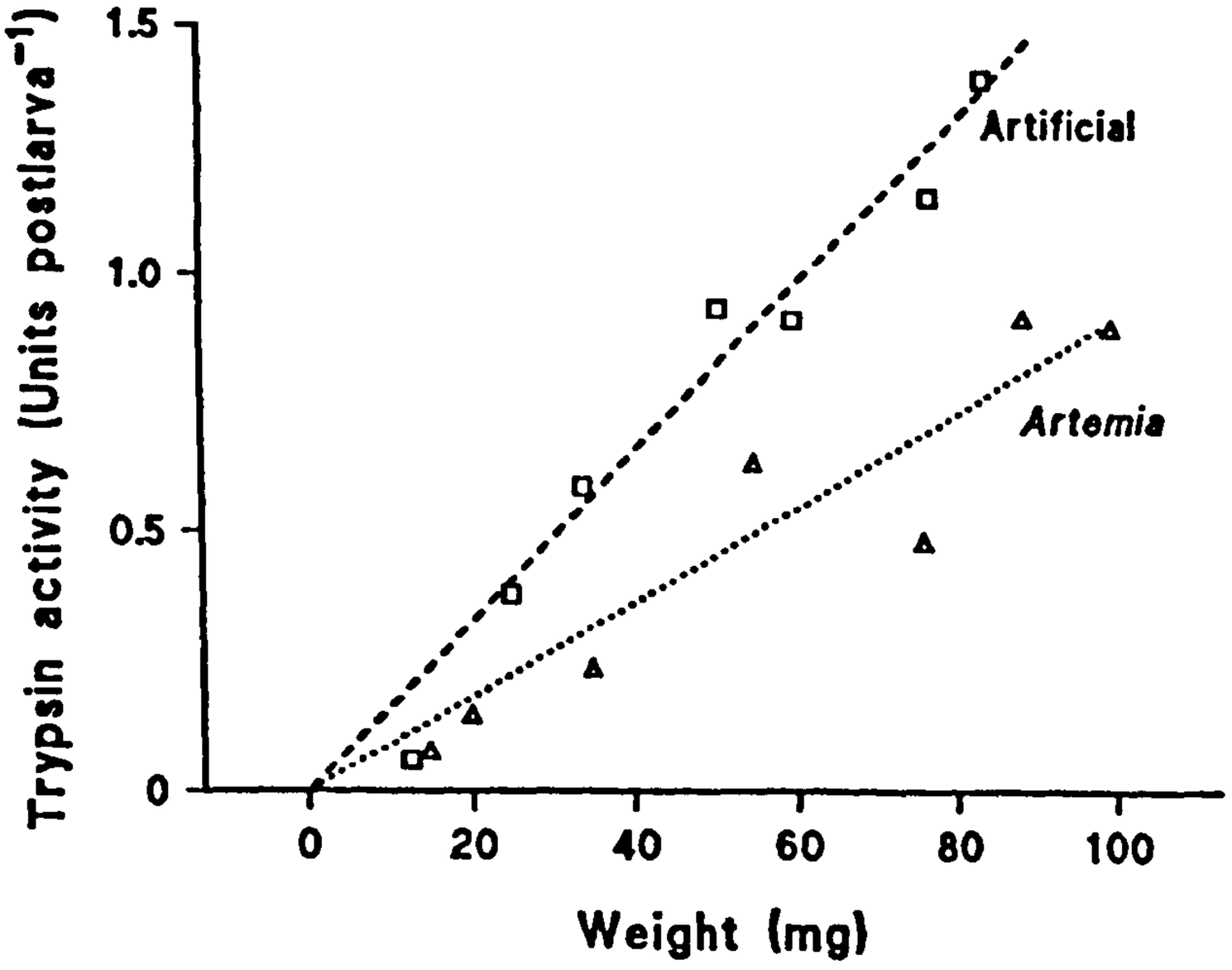


Figure 8a. Total protease activity (units/larva) in carp larvae reared on Artemia nauplii to five weight ranges and then either fed Artemia nauplii or fed Frippak CD3 artificial diet. Values are means ± s.d. (n = 3 to 6) for each point. Within each size range, treatments bearing different superscripts produced significantly different levels of protease activity ($p<0.05$).

Figure 8b. Plot of trypsin activity against total protease activity in carp larvae fed live and artficial diets, data taken from Figures 6a and 7a). Correlation of data sets ($r = 0.87$) excludes values for larvae larger than 12 mg, as indicated in the figure.

Figure 8a

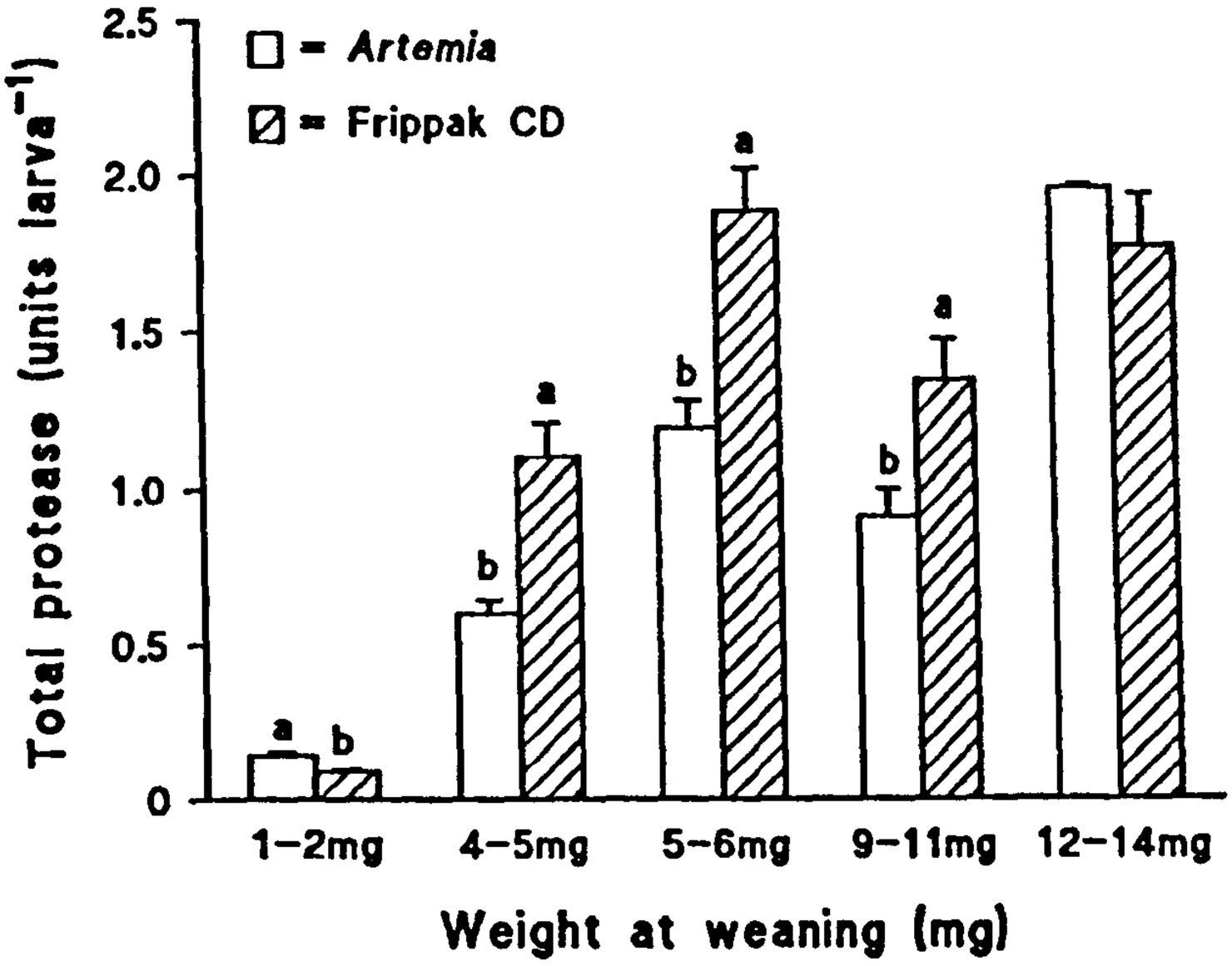


Figure 8b

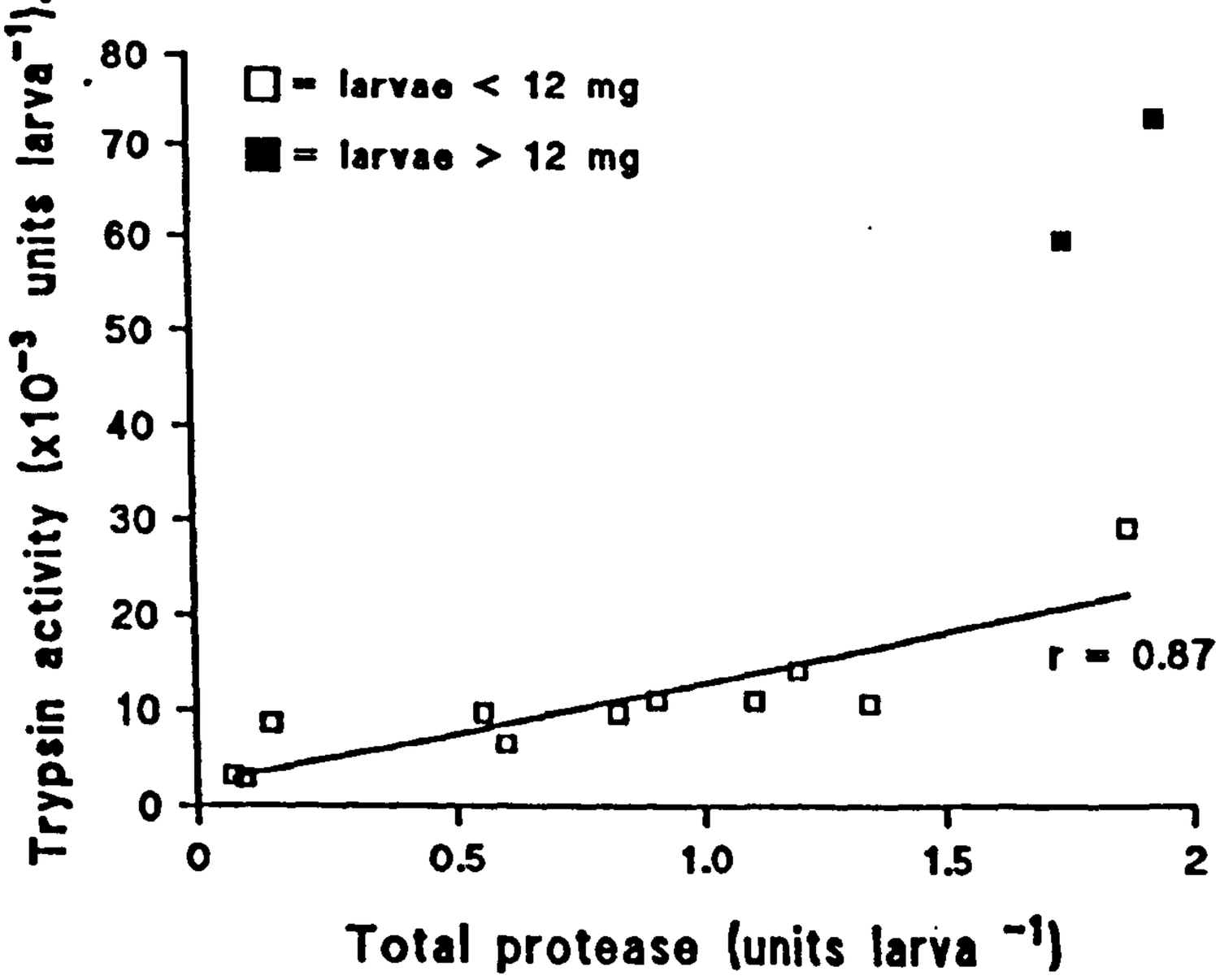


Figure 9a. Trypsin activity (x 10^{-3} Units $larva^{-1}$) in 2 mg carp larvae during transition from feeding on Artemia nauplii to Frippak CD3 and vice-versa. Values are means $\pm$ s.d., n = 6 to 9 for each point. Dotted lines mark times at which gut was completely filled with second diet.

Figure 9b. Trypsin activity (x 10^{-3} Units $larva^{-1}$) in 4 mg carp larvae during transition from feeding on Artemia nauplii to Frippak CD3. Values are means $\pm$ s.d., n = 6 to 9 for each point. Dotted lines mark times at which gut was completely filled with second diet.

Figure 9a.

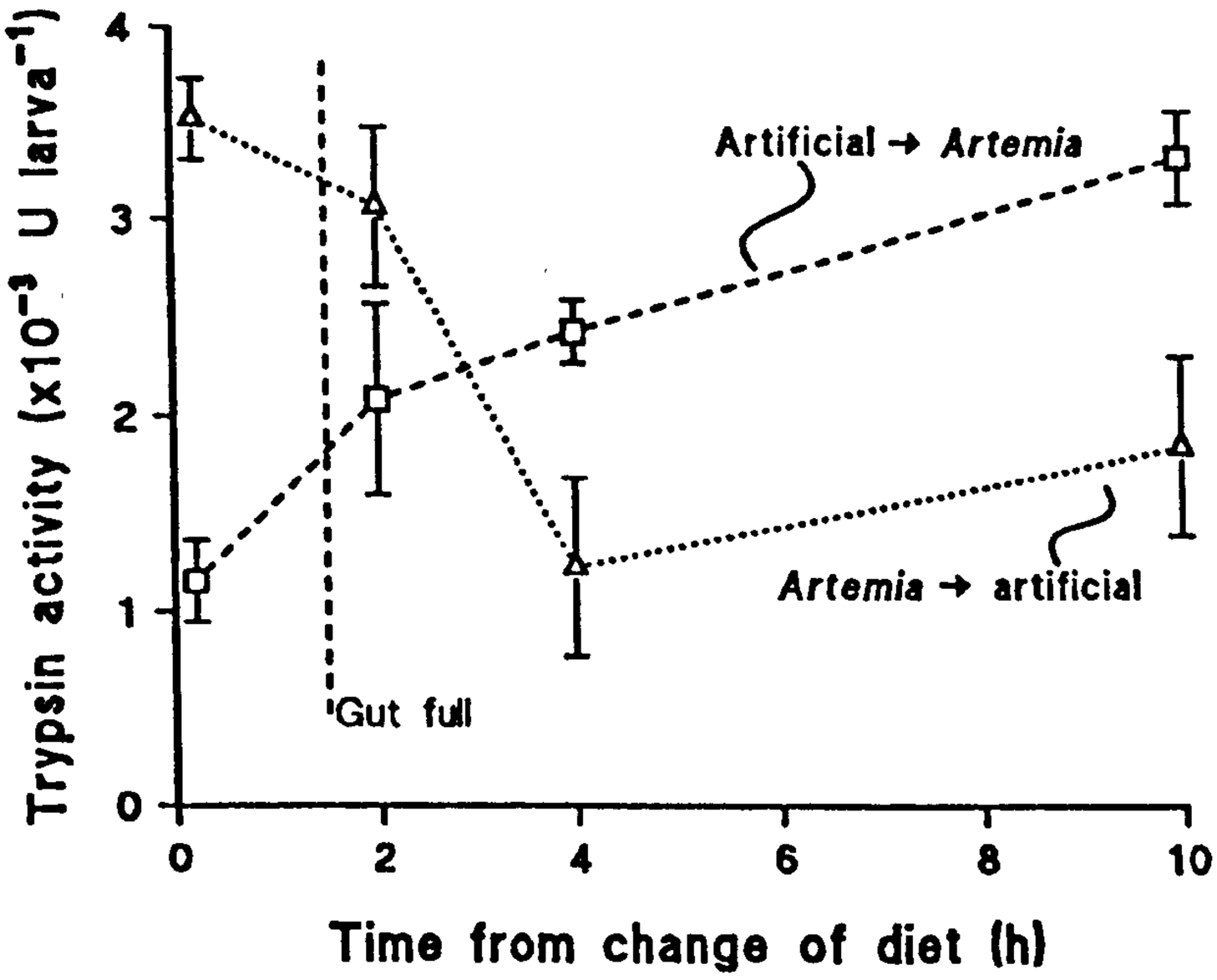


Figure 9b.

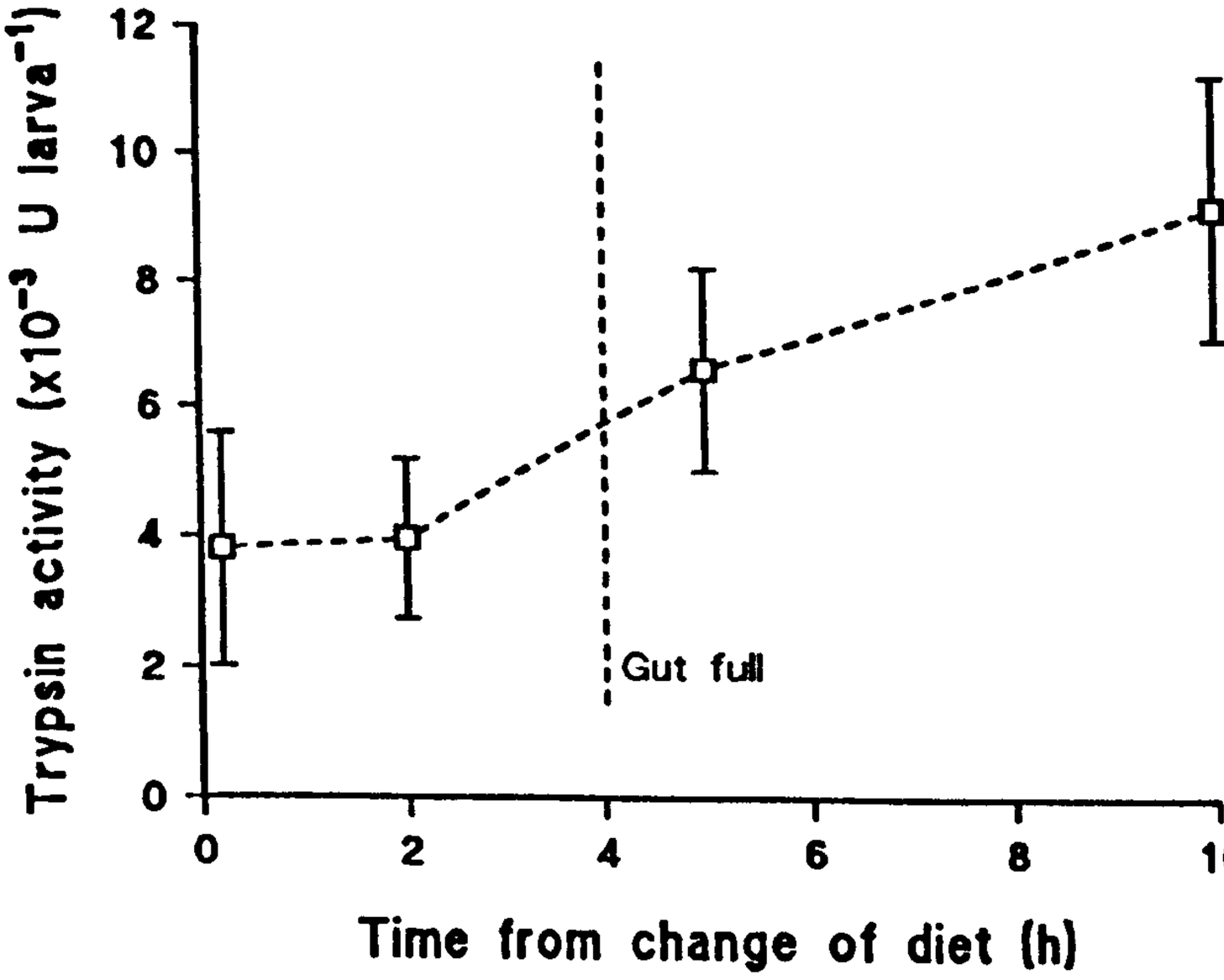


Plate 1. Photomicrograph showing physically intact Frippak CD microcapsules in the hind-gut of first-feeding carp larva.

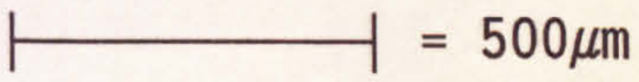


Plate 2a. Photomicrograph of cross-linked cod roe microcapsules after 12 h hydration in seawater at 20°C.

⊢——⊣ = 100 μm

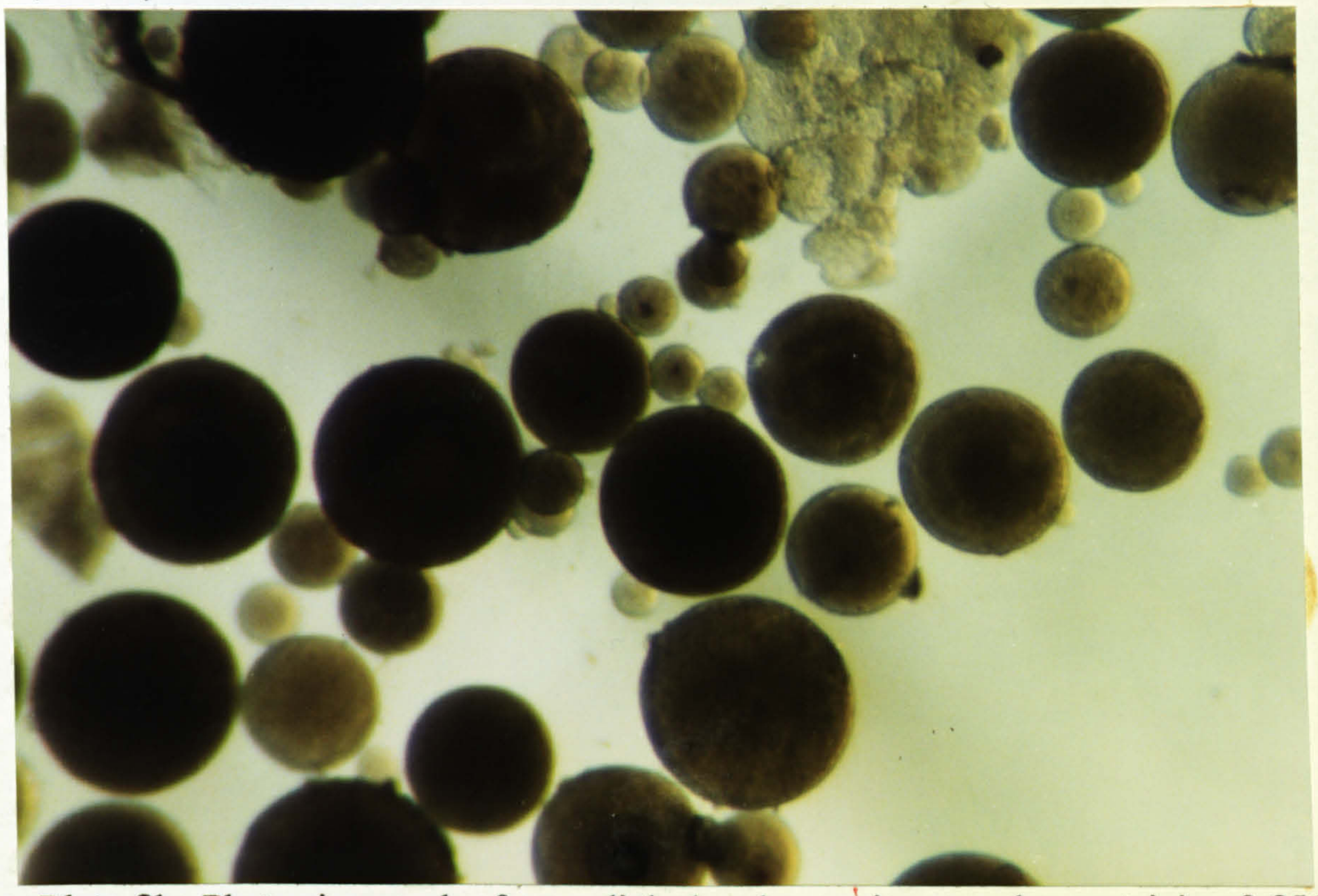


Plate 2b. Photomicrograph of cross-linked cod roe microcapsules containing 0.05% pancreatin after 2h hydration in seawater at 20°C.

⊢——⊣ = 100 μm

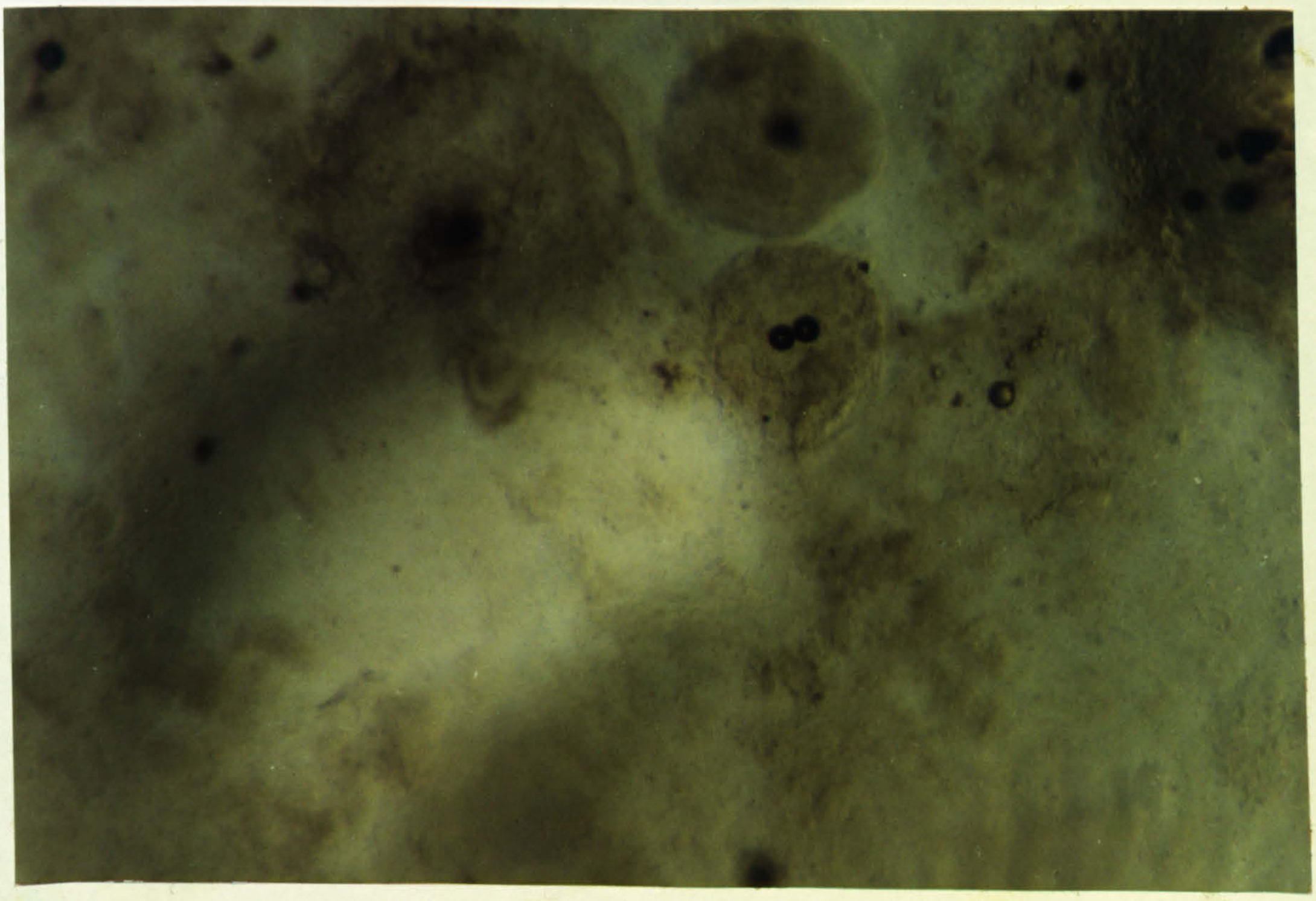


Plate 3a. Photomicrograph of cross-linked haemoglobin microcapsules after 12 h hydration in seawater at 20°C.

⊢——⊣ = 100 μm

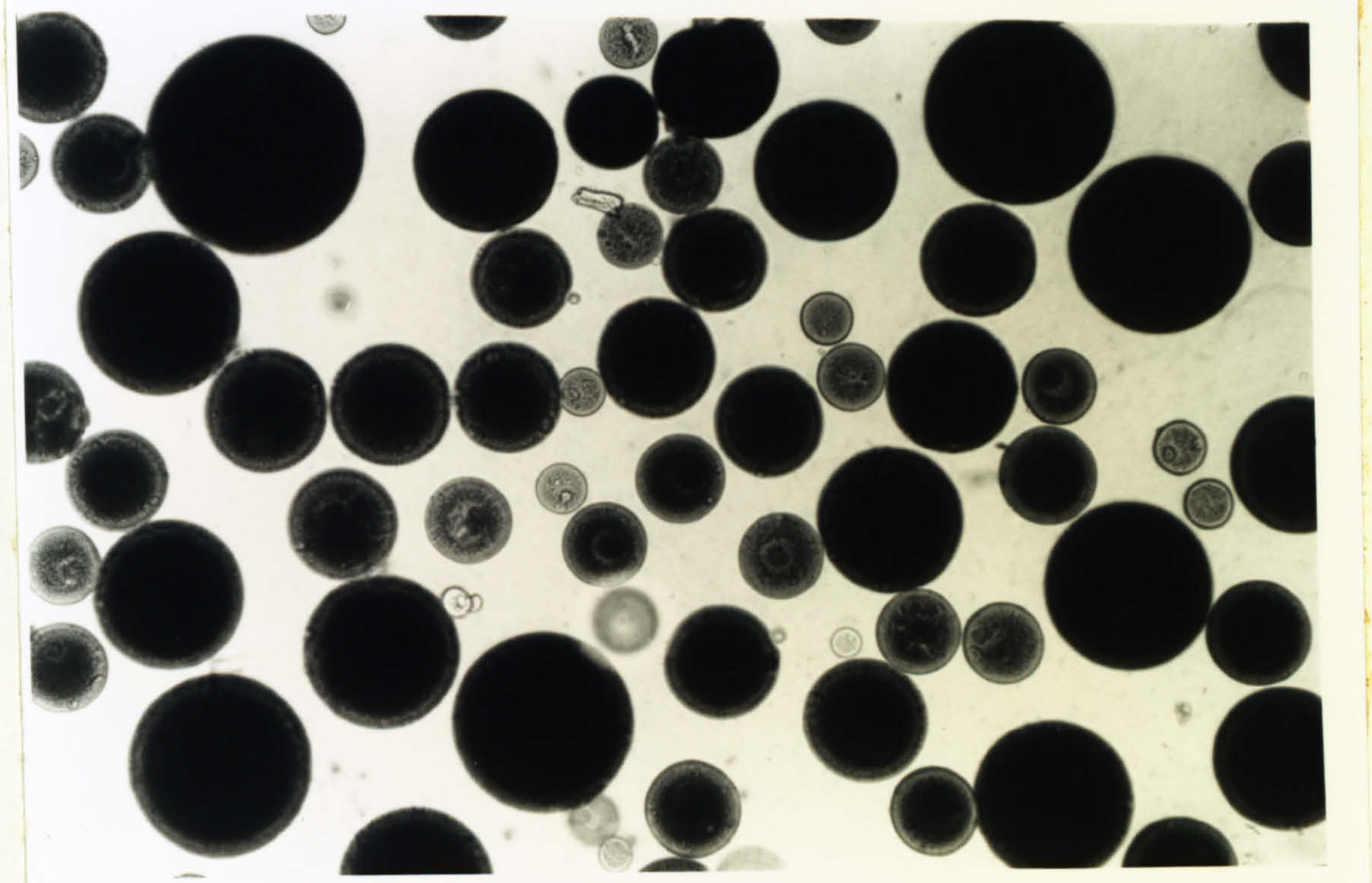


Plate 3b. Photomicrograph of cross-linked haemoglobin microcapsules containing 5% pancreatin after 1h hydration in seawater at 20°C.

⊢——⊣ = 100 μm

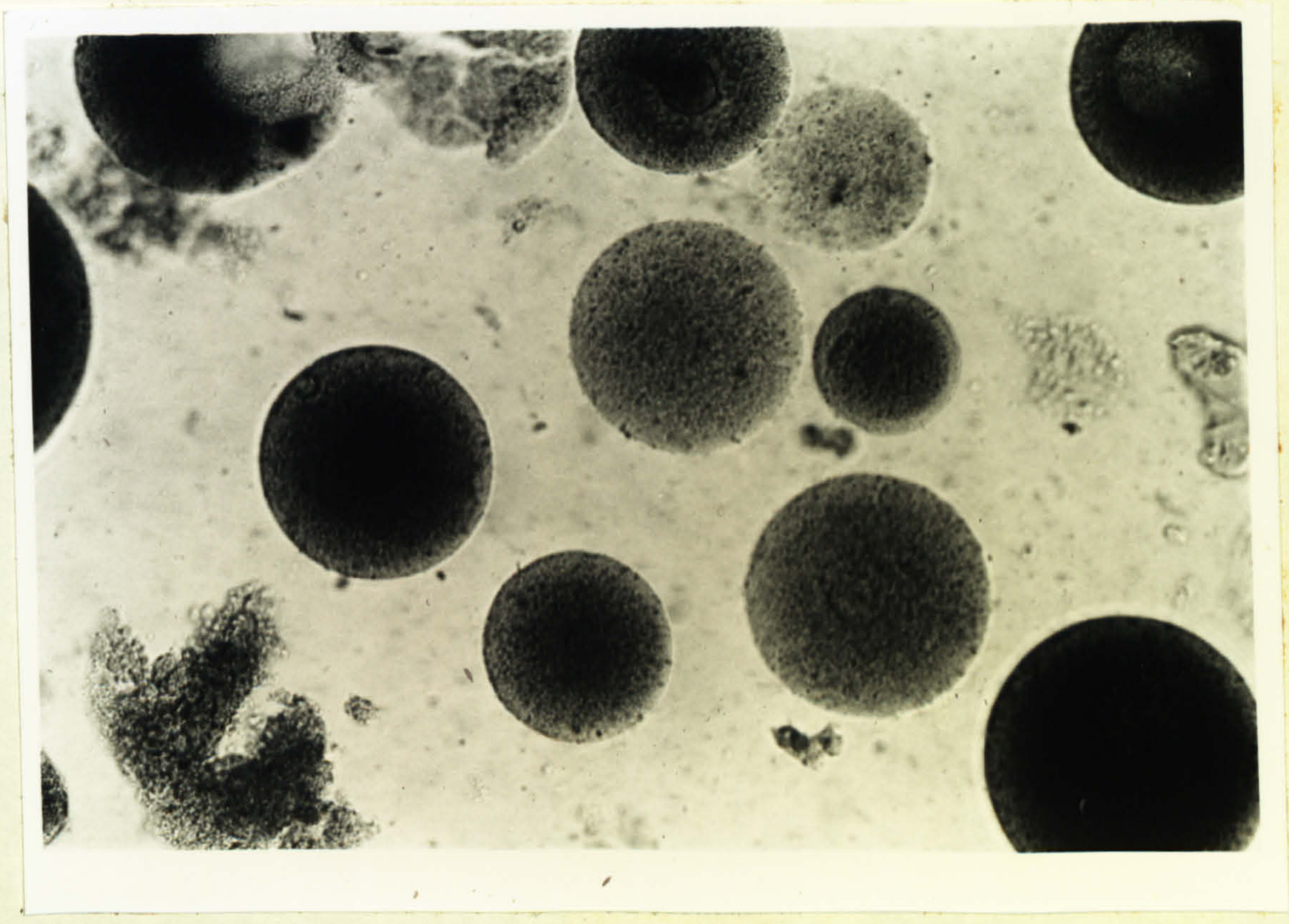


Plate 4a. Photomicrograph of cross-linked cod roe microcapsules in foregut of first-feeding carp larva.

⊢——⊣ = 500 μm

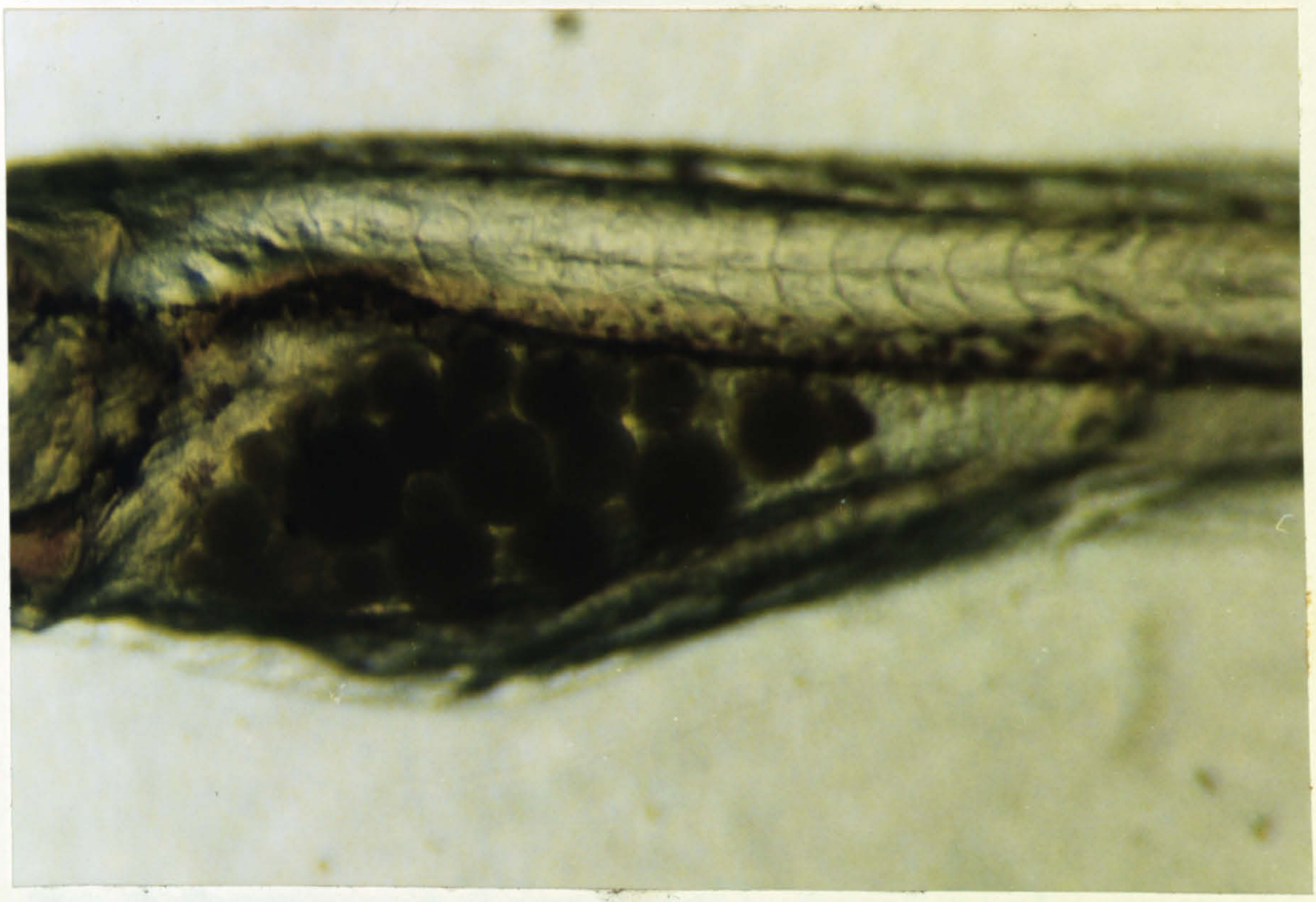


Plate 4b. Photomicrograph of cross-linked cod roe microcapsules containing 0.05% pancreatin in foregut of first-feeding carp larva carp larva. Arrow shows disintegrated capsule.

⊢——⊣ = 300 μm

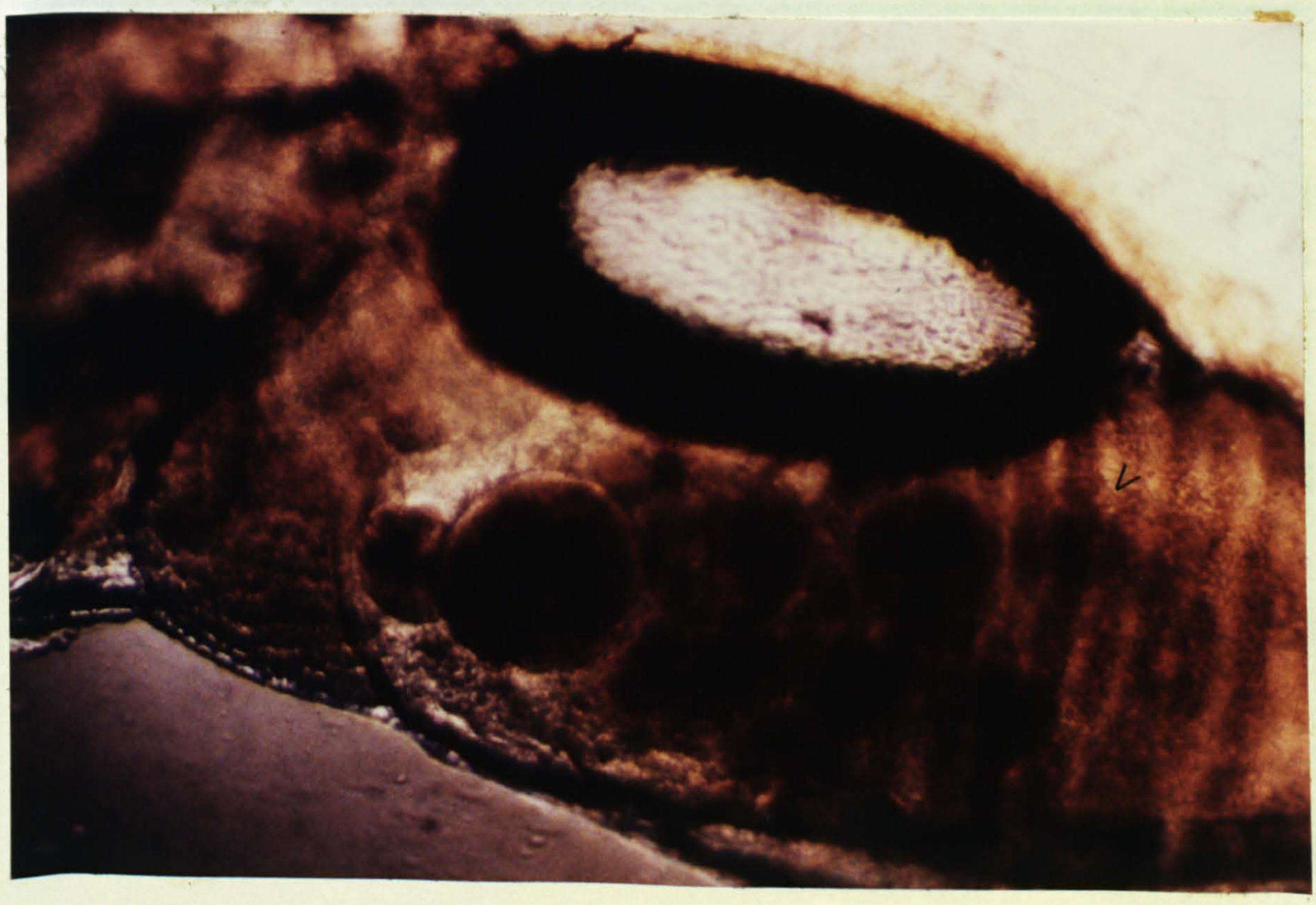


Plate 5a. Photomicrograph of remains of cross-linked haemoglobin microcapsules containing 5% pancreatin in foregut of first-feeding carp larva.

⊢——⊣ = 300 μm

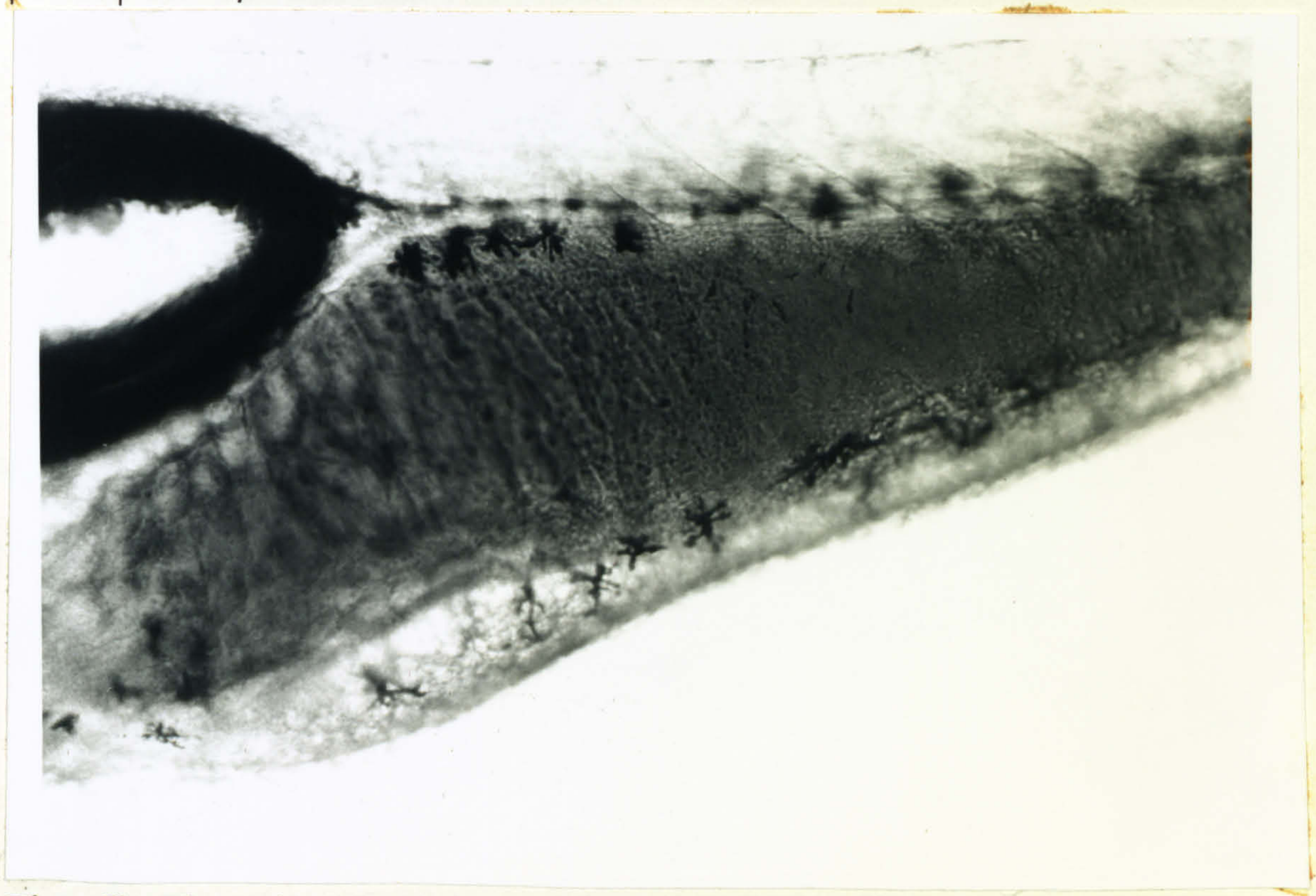
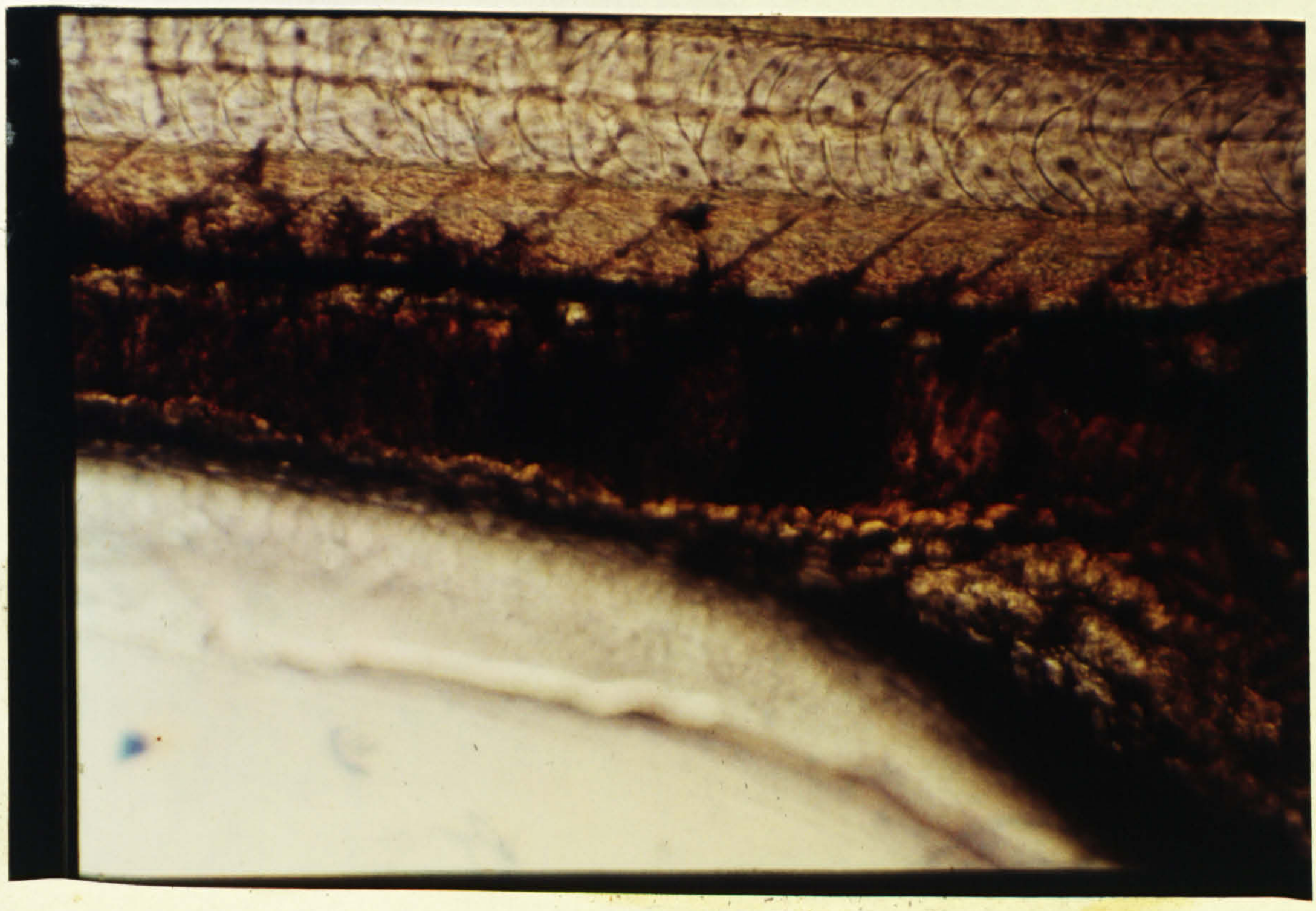


Plate 5b. Photomicrograph of contents of the hindgut of first-feeding carp larva after ingestion of a mixture of Frippak CD3 spray-dried diet and haemoglobin microcapsules containing 5% pancreatin.

⊢——⊣ = 300 μm



Chapter 4

BIOCHEMICAL COMPOSITION AND DIGESTIVE ENZYME ACTIVITY IN LARVAE AND POSTLARVAE OF PENAEUS JAPONICUS (BATE), DURING HERBIVOROUS AND CARNIVOROUS FEEDING.

Results presented in this chapter have contributed to the publications;

Le Vay, L., Rodríguez, A., Mourente, G. and Jones, D.A. (1993). Influence of diet on digestive activity and growth of Penaeus japonicus larvae - implications for nutritional studies. In; Carillo, M., Dahle, L., Morales, J, Sorgeloos, P, Svennevig, N and Wyban, J. (editors) Europ. Aquacult. Soc. Spec. Pub. 19. pp 145.

Rodríguez, A., Le Vay, L., Mourente, G. and Jones, D.A. (1994). Biochemical composition and digestive enzyme activity in larvae and post-larvae of Penaeus japonicus (Bate), during herbivorous and carnivorous feeding. Marine Biology 118: 45-53.

INTRODUCTION

Although Penaeus japonicus represents only 1% of global aquaculture production of penaeid prawns (Bailey-Brock and Moss 1992), the pioneering establishment of culture technology for this species by Hudinaga (e.g. Hudinaga 1942) was crucial to the development of penaeid culture as an international industry (Shigueno 1992). P. japonicus is principally cultured in Japan and Taiwan, with limited development in mediterranean Europe. Although Shigueno (1992) has estimated that production had risen to a maximum of only 3000t in 1987 per annum in Japan, the high value of fresh product has sustained interest in optimisation of culture methods, and particularly study of nutritional requirements and development of diets to replace or supplement live feeds in larval culture (see Chapter 1).

After the non-feeding nauplius stages, penaeid shrimp larvae develop through three herbivorous filter-feeding protozoeal (PZ_{1-3}) moults before the final mysis (M_1-M_3) stages prior to metamorphosis to postlarva. In the wild, penaeid larvae are found in coastal waters where the co-occurrence of both phytoplankton and zooplankton prey is typical. These conditions are mimicked in most commercial culture systems, where microalgal feeds are supplied at least until the end of the mysis stages, with the addition of zooplankton, usually Artemia nauplii, between PZ_3 and M_3 (Jones et al. 1987, Hirono and Leslie 1992, Liao 1992, Smith et al. 1992), although some authors recommend discontinuation of algal feeding after PZ_3 (Leger and Sorgeloos 1992). In Japan it is common hatchery practise to rear P. japonicus larvae in outdoor tanks (Shigueno 1992). Prior to stocking, diatoms (mainly Chaetoceros and Skeletonema species) are bloomed in the larval culture tanks and Artemia nauplii are usually added from the onset of the mysis stages, with no further addition of algae. Where artificial diets are used, they are usually added from early in the protozoeal stages.

Under laboratory conditions penaeid larvae usually begin raptorial feeding on zooplankton at the last protozoeal (PZ_3) stage, with a gradual decline in filter-feeding through the mysis stages to complete carnivory during the first post-larval moults (Emmerson 1980, 1984 for Penaeus indicus, Yufera et al. 1984 for Penaeus kerathurus). However Penaeus aztecus has been shown to feed successfully on phytoplankton throughout the post-larval period (Gleason and Zimmerman 1984). Laboratory studies have shown several penaeid species to be capable of attaining metamorphosis whilst feeding exclusively on phytoplankton (Gopalakrishnan 1976 for Penaeus marginatus, Kuban et al. 1985 for P. aztecus, P. setiferus, P. vannamei and P. stylirostris). In general, although survival rates are comparable, growth is superior in larvae receiving Artemia nauplii from the M_1 stage (Kuban et al. 1985).

While the complex larval development of penaeid prawns has hampered

research into their nutritional requirements, their free-swimming larval development has allowed detailed description of the ontogeny of digestive anatomy and physiology (Lovett and Felder 1989, 1990a,b, Abubakr and Jones 1992, Jones et al. 1993a, 1993b). The ability to grow and survive on such different food as phytoplankton or zooplankton implies a high degree of flexibility in digestive physiology to meet nutritional needs. There have been relatively few studies of digestive enzyme development in P. japonicus larvae. Laubier-Bonichon et al. (1977) report trypsin and amylase activity for most larval stages, and Galgani and Benyamin (1985) trypsin and proteases for some stages. More recent studies on the development of digestive physiology in P. setiferus and P. monodon larvae have described patterns of enzyme secretion attributed to changing functional morphology of the gut and the change in feeding habit during the mysis and early postlarval stages (Lovett and Felder 1989, 1990a,b,c, Abubakr and Jones 1992). Jones et al. (1993a,b) have found that, while underlying development of digestive enzyme secretion in P. monodon larvae may be genetically cued, there is also considerable diet-dependant modulation of activity, particularly in the mysis stages. In contrast, previous studies emphasised the importance of diet-independent development of digestive enzyme secretion (Lovett and Felder 1990a).

Since the development of effective artificial diets for penaeid larvae (Jones et al. 1979a), complete replacement of algae is now routine in some commercial hatcheries (Ottogalli 1991, 1993, Avale and Rothius 1991, see Chapter 1). However, it is apparent from recent reviews that, despite the widespread use of formulated feeds in commercial culture of penaeid larvae, there is urgent need for information on nutritional requirements for even the major dietary components; protein, lipid and carbohydrate during larval life (Guillaume 1990, Kanazawa 1990). Futhermore, virtually nothing is known of the relative importance of phytoplankton and zooplankton in fulfilling nutritional requirements.

This chapter describe the separate and interactive influences of herbivorous and zooplanktivorous feeding on the biochemical composition of P. japonicus larvae. The study set out to examine the role of algal food in larval nutrition during the mysis stages and changes in digestive enzyme in response to diet.

The specific objectives of the study were;

1. To analyse Artemia nauplii and C. gracilis for proximate composition and fatty acid content.

2. To compare growth and survival of P. japonicus mysis larvae fed either Artemia nauplii or C. gracilis, or a combination both.

3. To examine the effect of the above dietary treatments on the proximate tissue composition and fatty acid content of postlarvae.

4. To examine the effect of the above dietary treatments on digestive enzyme activity in P. japonicus larvae.

MATERIALS AND METHODS

This section describes methods used in an experiment carried out at the Instituto de Ciencias Marinas de Andalucia, Cádiz, Spain.

Algal culture

Chaetoceros gracilis was grown in u.v. irradiated, well-source sea water filtered to 1μm, salinity 32^{o}/oo, supplemented with 0.2ml l^{-1} commercial plant fertiliser (Nutrileaf, Agrocross S.A.) and 1ml l^{-1} sodium silicate solution (5.8% w/v). Culture densities reached $6x10^{6}$ cells ml^{-1} during semi-continuous batch culture. A concentration of 50 cells μl^{-1} was initiated in P. japonicus larval cultures, though further growth raised densities to up to 125 cells μl^{-1}. Samples were collected for biochemical analysis by centrifugation, washed, frozen in liquid nitrogen, lyophilized and stored at -80^{o}C (Mourente et al. 1990).

Artemia nauplii

Cysts of a parthenogenetic Artemia sp. strain, collected from the salt-marshes of Cádiz, Spain (Roman and Rodríguez 1989) were hatched overnight in 1μm filtered, u.v. irradiated, well-source water, salinity 32^{o}/oo. Fresh nauplii were added to larval cultures each day after water exchange. Samples of freshly hatched nauplii were also collected for biochemical analysis.

Culture of Penaeus japonicus larvae

P. japonicus larvae were obtained from a commercial hatchery (CUPIMAR S.A., San Fernando, Cádiz, Spain) during the spawning season May-June 1992. Larval stages were identified following Hudinaga (1942). Larvae were reared from PZ_1-PZ_3 using C. gracilis at a density of 50-125 cells μl^{-1} in a 500 l cylindrical fibreglass tank. Experimental rearing containers were 25 l clear acrylic tubes fitted with conical bases and lowest point aeration. Sea water was supplied from a well source, filtered to 1μm and u.v. irradiated. Water renewal was 50% day^{-1}. Temperature and salinity during the experiment were 26-27^{o}C and 32-33^{o}/oo respectively. A photoperiod of 16H light:8H dark was maintained throughout the experiment.

Experimental treatments

The growth, survival, digestive enzyme activity and biochemical composition of P. japonicus postlarvae reared through the mysis stages using three feeding regimes were studied. From M_1 larvae were stocked at 100 ml^{-1} into experimental rearing containers, with three tubes allocated to each of the following feeding regimes;

(A) C. gracilis at 50-125 cells μl^{-1} plus freshly hatched Artemia nauplii at 3-7 ml^{-1}.

(B) Freshly hatched Artemia nauplii at 3-7 ml^{-1}.

(C) C. gracilis at 50-125 cells μl^{-1}.

Sampling

Samples of larvae were collected at each mysis stage for enzyme analysis. When more than 50% of animals had reached PL_1, cultures were harvested and counted. Samples of PL_1 stage animals were sorted and collected for dry weight estimation, and biochemical analysis.

Dry weight determination

Samples of 15-25 postlarvae were washed in distilled water and oven dried at 110°C for 24h. After cooling for at least 1h, samples were weighed using a Mettler UM3 microbalance.

Total lipid extraction and quantification

Lipids were extracted after homogenization in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene (BHT) (Folch et al. 1957) and dried in oxygen-free nitrogen. Total lipid content was determined gravimetrically using a Mettler UM3 microbalance.

Fatty acid analysis

Fatty acid methyl esters were prepared by acid-catalysed transmethylation for 16h at 50°C, using nonadecanoic acid (19:0) as internal standard (Christie 1989). Methyl esters were extracted and purified following the method of Tocher and Harvie (1988). The fatty acid methyl esters were analysed in a Hewlett-Packard 5890 A Series II gas chromatograph equipped with a chemical bonded (PEG) Omegawax 320 fused silica wall coated capillary column (30m X 0.32mm i.d., Supelco Inc., Bellefonte, USA) using hydrogen as the carrier gas with a thermal gradient from 185°C - 235°C. Individual fatty acid methyl esters were identified following the methods of Tocher and Harvie (1988) and quantified using a Hewlett-Packard 3394 recording integrator.

Proximate compositional analysis of feeds and postlarvae

Nitrogen content analyses of postlarvae and feeds were performed in an elemental

analyser Carlo Erba 1106, using cyclohexane 2,4-dinitrophenylhydrozone as standard. Protein content was obtained by multiplying nitrogen content by 6.25. Carbohydrate was determined by the phenol-sulphuric acid method (Kochert 1978). Energy values for C. gracilis and Artemia nauplii were obtained by multiplying carbohydrate, protein and lipid contents by 17.14, 23.42 and 39.31 J respectively (Winberg 1971).

Trypsin activity

Samples of larvae and postlarvae were homogenised in 1.5 ml ice-cold Tris-HCl buffer (46 mM Tris, 11.5 mM $CaCl_2$, pH 8.1). Homogenates were centrifuged at 12,000 x g for 5 min and the supernatant was assayed for trypsin-like activity at 25°C using 1 mM Nα-p-toluenesulphonyl-L-argenine methyl ester (TAME) in the same buffer (Hummel 1959). Reaction volume was 1000μl, using 200μl of sample and 100μl of 10mM TAME. The rate of hydrolysis of the substrate was recorded as increase in absorbance at 247 nm in a Perkin-Elmer Lambda UV/vis spectrophotometer. Activity per larva was calculated as described in Chapter 3 and expressed in units of μmol of substrate cleaved per minute (Rick 1984).

Amylase activity

Samples of larvae and postlarvae were homogenized in 1.5 ml ice-cold phosphate buffer (20 mM phosphate, 10 mM NaCl, pH 6.9), centrifuged for 5 min at 12,000 x g and 375μl of the supernatant incubated with 375μl 1% w/v starch in the same phosphate buffer. Production of reducing sugars was quantified colorimetrically following addition of 750μl dinitrosalicylic acid reagent (1% 3,5-dinitrosalicylic acid, 30% w/v potassium sodium tartrate) (Rick and Stegbauer 1984). A standard curve was prepared using maltose solution instead of enzyme sample, and results expressed as μmol of maltose equivalents released per larvae per minute.

Statistical analysis

Differences between treatments were analysed by one-way analysis of variance, followed by a multiple comparison test (Tukey's for equal sample sizes, Scheffé's method for unequal sample sizes). Differences are reported as statistically significant when $p<0.05$.

RESULTS

Composition of feeds

The proximate composition, energy content and fatty acid content of C. gracilis and Artemia nauplii are shown in Table 1. The differences in protein, lipid and energy content of the feeds are largely accounted for by the high ash content of C. gracilis (73.8%) compared to that of Artemia nauplii (9.8%). Protein content of C. gracilis

is still substantially lower (26.3%) than that of Artemia nauplii (61.6%) when expressed as percentage of ash-free dry weight. Differences in highly unsaturated fatty acid (HUFA) content are also largely accounted for by the difference in ash content, although only C. gracilis contained measurable amounts of 22:6n-3. In contrast, the similar levels of carbohydrate found in the two feed species reflect a higher content in the diatom compared to the Artemia nauplii when expressed as percentage of ash-free dry weight.

Size and survival at postlarva

Length, weight, survival and proximate analysis at PL_1 are shown in Table 2. There was no significant difference in dry weight, length or survival between the two treatments receiving either C. gracilis or Artemia nauplii as sole feed from M_1 to PL_1. However, postlarvae reared on the mixed diet were significantly greater in dry weight, length and survival compared to both other treatments ($p<0.05$).

Biochemical composition of postlarvae

Table 2 also shows proximate tissue composition of samples of postlarvae from the three treatments. Postlarvae from the two treatments receiving C. gracilis or Artemia nauplii as sole food from did not differ significantly in body protein or lipid content. Carbohydrate content was significantly higher for the treatment receiving only C. gracilis than the other treatments. Postlarvae from the treatment receiving both C. gracilis and Artemia nauplii had significantly higher body protein content and lower lipid content than the treatments receiving only one feed type ($p<0.05$).

Fatty acid contents of postlarvae are shown in Table 3. Comparison of the treatments receiving either Artemia nauplii or C. gracilis exclusively shows that the fatty acid content of the postlarvae reflects the content of these feeds. The n-3 HUFA content of postlarvae reared on the mixed feed was intermediate to that of animals reared on either feed exclusively. The 20:5n-3 content of animals from the mixed feed treatment did not differ significantly from that of animals reared on Artemia nauplii alone, while n-6 HUFA content was identical to that of animals reared on C. gracilis. Despite the lack of a measurable quantity of 22:6n-3 in Artemia nauplii, 1.2 $\mu g\ mg^{-1}$ of this fatty acid was recorded in postlarvae fed Artemia nauplii exclusively during the mysis stages. In contrast, the presence of low levels of 22:6n-3 in C. gracilis resulted in significantly more of this fatty acid accumulating in animals feeding on the alga alone. Surprisingly, there was no difference between the 22.6n-3 content of animals feeding on the mixed diet and those feeding only on Artemia nauplii.

Digestive enzyme activity

Results of trypsin analyses are shown in Figure 1. After a steady rise in trypsin activity from PZ_1 to M_1 in larvae feeding on C. gracilis, there are significant differences in trypsin activity between treatments during the mysis stages. Activity in mysis larvae and postlarvae feeding on C. gracilis was substantially higher than that in larvae feeding on Artemia nauplii. Trypsin activity in M_2 and M_3 larvae feeding on C. gracilis was over five times higher than in larvae feeding on Artemia nauplii. Trypsin activity in larvae feeding on the mixed diet followed an intermediate pattern to those observed in larvae feeding on C. gracilis or Artemia nauplii alone.

Results of amylase analyses are shown in Figure 2. There are significant differences in amylase activity between dietary treatments in the mysis stages, but these are not as large or as consistent as those observed for trypsin. At M_1 amylase activity in larvae feeding on C. gracilis was double that measured in larvae feeding on Artemia nauplii or the mixed diet. At M_2 activity in larvae feeding on C. gracilis was almost three times that in larvae feeding on Artemia nauplii, with the mixed feed producing intermediate activity. At M_3 activity in larvae feeding on C. gracilis fell to the same level as observed in larvae feeding on Artemia nauplii alone, with the mixed diet producing significantly higher activity ($p<0.05$). Amylase activity in animals from the treatment feeding on Artemia nauplii alone did not rise substantially until PL_1, where levels were equivalent to those for animals feeding on C. gracilis, both these treatments giving higher values than the mixed diet.

DISCUSSION

Biochemical composition of Artemia and C. gracilis

The proximate analysis of Artemia nauplii is the range of values for nauplii used in penaeid larval culture (see Table 4, Chapter 1). The high ash content of C. gracilis is surprising, though there are few data in the literature for composition of this species. However, repeated analyses of new cultures from the same stock sub-culture used in this study have yielded identical results to those presented here and results of proximate analysis of the organic component of C. gracilis are in general agreement with results of Ben-Amotz et al. (1987) for this species and values for other algal species used in penaeid larval culture (Jones et al. 1993b). Some error may have been introduced into the estimation of protein from nitrogen content by the use of a conversion factor of 6.25, which is derived from fish proteins (Jobling 1983). However, the calculated value (7% of dry weight) is not greatly different from total protein content of C. gracilis calculated from amino-acid composition (12% of dry weight, Brown 1991). By comparison, ash content of C. calcitrans can be as high as

48.4% (Tobias-Quinitio and Villegas 1982), with protein content as low as 17.6%. (Utting 1986).

Whilst a review of the literature indicates a dietary protein requirement for P. japonicus larvae of 45% (Table 5, Chapter 1), it is common practise to use C. gracilis as the main algal species for the culture of this and other penaeid species (Forbes 1992, Liao 1992, Smith et al. 1992) and Chaetoceros species are known to form a major component of the diet of penaeid larvae in the wild (Preston et al. 1992). Although the essential dietary requirement for n-3 HUFA by P. japonicus larvae is known (Jones et al. 1979b), no absolute quantitative requirement has been determined (D'Abramo 1990). The levels of n-3 HUFA recorded in C. gracilis fall below the 1% level recommended by Guillaume (1990) for P. japonicus larvae. While the level of 20.5n-3 in Artemia nauplii used in the present study is certainly adequate, the significance of an absence of 22.6n-3 is unknown, as previous studies on the influence of dietary HUFA on growth of larval and juvenile penaeids have not compared the relative importance of 20.5n-3 and 22.6n-3 (see D'Abramo 1990 for review).

Present survival of P. japonicus mysis larvae feeding on C. gracilis alone and on the mixed feeding regime are within the range of results reported by Hudinaga (1942) for similar dietary regimes. While length of postlarvae from the mixed feeding regime is equal to the maximum recorded by Hudinaga (1942), the length of postlarvae reared during the mysis stages on either Artemia nauplii or C. gracilis falls below the minimum values recorded in the same study.

In general, successful feeds for penaeid larvae have ranged from 23-55% protein content and HUFA content greater than 1% (see Table 5, Chapter 1). In the present study an algal diet of less than 7% protein content and low HUFA content supported survival and growth through the mysis stages equal to that achieved with a zooplankton diet of higher HUFA and protein content. Given that prior to the experiment all larvae had been raised from PZ_1 exclusively on the same algal feed, it is clear that the absolute requirements of P. japonicus larvae for protein and HUFA may be substantially lower than suggested in the literature. However, results with the mixed feed regime show that while survival and growth through the mysis stages is possible on either C. gracilis or Artemia nauplii alone, these diets are sub-optimal.

Although a trend towards carnivory during the mysis stages has been reported for larvae of several penaeid species (Emmerson 1984, Yufera et al. 1984, Chu and Shing 1986, Kurmaly et al. 1989b), larvae are capable of ingesting similar quanitities of microalgae or Artemia nauplii through this stage of development (Emmerson 1984 for Penaeus indicus, Kurmaly et al. 1989b for Penaeus monodon). The development and growth of P. japonicus larvae from M_1 to postlarvae feeding

either on C. gracilis or Artemia nauplii alone is evidence of a similarly facultative feeding habit in this species. However, it is remarkable that the large difference in gross nutritional content of C. gracilis and Artemia nauplii, and consequent differences in energy and protein intake, is not reflected in the growth, survival or body composition of the P. japonicus larvae feeding on them. The explanation for this may lie in the digestive response of the larvae to the two feeds.

Enzymatic digestion is of particular importance in the larvae of penaeid species. The hepatopancreas is not fully functional until sometime after metamorphosis, and the anterior midgut diverticulae function as the main site of digestive enzyme secretion during larval development (Jones 1988, Lovett and Felder 1989, 1990b, Abubakr and Jones 1992). In compensation for feeding on an algal species of low protein content, the high level of trypsin activity observed in P. japonicus larvae presumably acts to maximise assimilation of this scarce nutrient, as has been suggested for Calanus helgolandicus (Harris et al. 1986). Furthermore, Jones et al. (1993a,b) report a stimulation of trypsin activity in P. monodon larvae by microalgae which does not appear to be related to gross dietary protein content. The higher levels of trypsin activity recorded in larvae feeding on C. gracilis in the present study may due to be the combination of a general response to low dietary protein availability and a direct stimulation of secretion by an active component of the alga.

In contrast, despite the relatively high levels of carbohydrate recorded in the C. gracilis, amylase activity was generally higher in larvae feeding on the alga than in those feeding on Artemia. However, there is some doubt as to ability of penaeid larvae to assimilate algal carbohydrates (Lovett and Felder 1989). Kristensen (1981) has found that structural polysaccharides, with the exception of chitin, are not readily digested by marine invertebrates - in contrast to the high digestibility of storage polysaccharides such as glycogen and amylose. In diatoms, up to 50% of total polysaccharide may occur in mannose-rich polymers associated with the siliceous frustule of the cell wall (Handa and Yanagi 1969, Hecky et al. 1973, Brown 1991) and the occurrence of high levels of mannose-rich polymers has been linked with the low digestibility of the alga Phaeodactylum cornutum (Epifanio 1983, cited in Brown 1991). Samain et al. (1985) observed high levels of amylase in Artemia fed starch-free diets, and a similar response may have occurred in the present study as a result of low availability of digestible carbohydrate in C. gracilis.

The superior larval growth, survival and protein retention in P. japonicus larvae receiving a mixture of Artemia nauplii and C. gracilis may be due to enzyme levels which result in improved digestion and assimilation of the Artemia component of the diet. Thus, while growth of larvae feeding on C. gracilis alone was very likely directly limited by nutrient availability, for larvae feeding on Artemia nauplii

assimilation and retention of dietary protein may have been limiting in the absence of the digestive enzyme response induced by the algal co-feed.

A peak in digestive enzyme production has been reported in PZ_3-M_1 larvae of several penaeid species (Laubier-Bonichon et al. 1977, Galgani and Benyamin 1985, McDonald et al. 1989, Lovett and Felder 1990a, Jones et al. 1993a, 1993b, Le Moullac et al. 1993). This period of development coincides with both the maximal volume of the anterior midgut diverticulae (AMD) (Lovett and Felder 1989, 1990b, Abubakr and Jones 1992), and also the onset of the transition in feeding habit from herbivorous filter-feeding to raptorial carnivory. Thus the decline in digestive enzyme activity from the early mysis stages of penaeid larvae has been interpreted either as an adaption to change in feeding requirements (Laubier-Bonichon et al. 1977) or as a consequence of regression of the AMD in the ontogenetic development of the digestive secretory apparatus (Lovett and Felder 1990a,c). In the present study there is evidence of great flexibility in digestive response to diet type through the later larval stages and it is clear that the patterns of trypsin and amylase activity in mysis stage penaeid larvae reported in the literature are likely to reflect adaptation the feeding regime used rather than the actual secretory capacity of the species in question.

High amylase:protease (A/P) ratios have been suggested to be indicative of omnivorous or herbivorous feeding habit in decapods and copepods, with low values typical of carnivores (Sather 1969, Laubier-Bonichon et al. 1977, Gaudy and Boucher 1983). In the present study, the large differences observed A/P ratios for different dietary treatments are primarily a consequence of the variation in trypsin activity and support the suggestion that decapod larvae capable of herbivorous feeding should exhibit independant regulation of these enzymes (Hirche and Anger 1987). In contrast carnivorous larvae such as Hyas araneus can exhibit positive correlation between trypsin and amylase activities within moult stages (Hirche and Anger 1987).

The degree to which the modulation of digestive enzyme activity in response to diet observed in this study extends through the 'critical' early postlarval period (Wickins 1976, Bages and Sloane 1991), when the AMD becomes vestigial, remains to be examined. Ingestion of benthic microalgae is known to persist through this period for some species (Gleason and Zimmerman 1984, Gleason 1986) and Griffith et al. (1992) have recently reported beneficial effects on growth and survival of Penaeus vannamei postlarvae feeding on benthic diatoms in addition to Artemia nauplii.

Table 1. Proximate composition (dry weight percentage), energy (J mg^{-1}) and fatty acid content (μg fatty acid mg^{-1}) of the diatom Chaetoceros gracilis and nauplii of Artemia (strain from the salt marshes of Cádiz, Spain) used for rearing Penaeus japonicus larvae.

	C. gracilis	Artemia
Protein	6.9 ± 0.5	56.2 ± 1.5
Lipid	4.1 ± 0.7	17.5 ± 1.1
Carbohydrate	15.2 ± 0.8	16.5 ± 0.3
Ash	73.8 ± 0.2	9.8 ± 0.4
Energy content	5.8 ± 0.2	21.3 ± 0.3
Fatty acid		
14:0	1.5 ± 0.1	2.2 ± 0.2
15:0	0.4 ± 0.1	2.4 ± 0.3
16:0	1.0 ± 0.1	18.9 ± 0.4
16:1n-9	1.1 ± 0.1	n.d.
16:1n-7	3.8 ± 0.4	22.2 ± 0.3
16:2	1.6 ± 0.2	1.9 ± 0.2
16:3	3.6 ± 0.2	2.1 ± 0.2
16:4	0.5 ± 0.1	0.5 ± 0.2
18:0	0.2 ± 0.0	6.2 ± 0.3
18:1n-9	0.1 ± 0.0	26.1 ± 0.6
18:1n-7	0.1 ± 0.0	17.8 ± 1.1
18:2n-6	0.1 ± 0.0	6.0 ± 0.5
18:3n-3	0.2 ± 0.0	4.2 ± 0.3
18:4n-3	0.1 ± 0.0	1.3 ± 0.1
20:1n-9	0.1 ± 0.0	0.3 ± 0.2
20:2n-6	0.1 ± 0.0	0.4 ± 0.0
20:3n-3	0.1 ± 0.0	0.3 ± 0.1
20:4n-6	0.3 ± 0.0	2.6 ± 0.2
20:4n-3	0.1 ± 0.0	0.5 ± 0.1
20:5n-3	4.6 ± 0.1	14.6 ± 0.6
22:6n-3	0.3 ± 0.0	n.d.
Total saturates	3.2 ± 0.2	30.5 ± 0.3
Total monones	5.3 ± 0.1	66.9 ± 2.1
Total polyunsaturates	11.9 ± 0.2	35.1 ± 0.3
n-6 HUFA	0.3 ± 0.0	2.6 ± 0.3
n-3 HUFA	5.1 ± 0.4	15.4 ± 0.5

Data are means ± s.d. (n=3), and s.d. = 0.0 implies an s.d. <0.05.
n.d. = not detected. HUFA = highly unsaturated fatty acids ($\geq C_{20}$ and with at least 3 double bonds).

Table 2. Protein, lipid and carbohydrate contents (expressed as μg mg^{-1} ash-free dry weight), dry weight and ash contents (expressed as μg $postlarva^{-1}$), length (mm) and survival (%) of Penaeus japonicus postlarvae reared using (A) Chaetoceros gracilis during all larval stages plus Artemia nauplii from mysis 1 stage, (B) C. gracilis until mysis 1 and only Artemia nauplii from mysis 1 to postlarva, and (C) only C. gracilis through all larval stages.

	(A)	(B)	(C)
Protein (μg mg^{-1})	695.7 ± 12.2^a	620.8 ± 29.3^b	594.5 ± 4.1^b
Lipid (μg mg^{-1})	263.2 ± 14.4^a	334.8 ± 10.2^b	349.3 ± 7.9^b
Carbohydrate (μg mg^{-1})	44.6 ± 1.7^a	43.3 ± 1.6^a	52.5 ± 3.5^b
Dry weight (μg $postlarva^{-1}$)	115.4 ± 6.8^a	76.3 ± 7.2^b	71.3 ± 6.7^b
Ash (μg $postlarva^{-1}$)	23.6 ± 3.1	16.4 ± 2.5	22.0 ± 2.3
Length (mm)	5.0 ± 0.2^a	4.3 ± 0.2^b	3.8 ± 0.1^b
Survival (%)	75.7 ± 5.3^a	46.0 ± 2.7^b	46.2 ± 3.8^b

Data are means ± s.d. (n=3). Values within a given row not bearing the same superscript are significantly different at $p<0.05$. If no superscript appears, values are not significantly different.

Table 3. Total lipid fatty acid contents (μg fatty acid mg^{-1} dry weight) of Penaeus japonicus postlarvae fed on (A) Chaetoceros gracilis during all larval stages plus Artemia nauplii since mysis 1 stage, (B) C. gracilis until mysis 1 and only Artemia from mysis 1 to postlarva, and (C) only C. gracilis through all larval stages.

Fatty acid	(A)	(B)	(C)
14:0	1.8 ± 0.2	1.6 ± 0.9	2.4 ± 0.6
15:0	3.3 ± 0.4	2.9 ± 0.8	4.8 ± 0.7
16:0	8.3 ± 0.2^{a}	9.9 ± 0.4^{b}	7.8 ± 0.3^{b}
16:1n-7	3.8 ± 0.2^{a}	4.9 ± 0.6^{a}	2.0 ± 0.1^{b}
16:2	0.7 ± 0.1	0.9 ± 0.5	0.5 ± 0.1
16:3	1.2 ± 0.1^{ab}	0.9 ± 0.1^{a}	1.5 ± 0.3^{b}
16:4	0.9 ± 0.0	1.2 ± 0.1	0.9 ± 0.0
18:0	6.5 ± 0.3	7.9 ± 0.5	5.8 ± 0.3
18:1n-9	7.9 ± 0.4^{a}	11.7 ± 0.8^{b}	2.6 ± 0.6^{c}
18:1n-7	8.2 ± 0.5^{a}	11.6 ± 0.6^{b}	2.5 ± 0.4^{c}
18:2n-6	2.1 ± 0.1^{a}	3.6 ± 0.1^{b}	0.8 ± 0.2c
18:3n-3	0.4 ± 0.1	0.9 ± 0.5	1.0 ± 0.2
18:4n-3	0.8 ± 0.3	1.1 ± 0.5	0.3 ± 0.1
20:0	0.3 ± 0.0^{a}	0.3 ± 0.1^{ab}	0.5 ± 0.1^{b}
20:1n-9	0.5 ± 0.0^{a}	0.7 ± 0.1^{a}	0.9 ± 0.1^{b}
20:1n-7	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
20:2n-6	0.3 ± 0.1	0.3 ± 0.0	0.5 ± 0.2
20:3n-6	0.2 ± 0.1^{a}	0.4 ± 0.0^{a}	0.7 ± 0.1^{b}
20:4n-6	2.1 ± 0.1^{a}	2.5 ± 0.1^{b}	0.9 ± 0.1^{c}
20:3n-3	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.2 ± 0.0^{a}	0.4 ± 0.0^{ab}	0.6 ± 0.2^{b}
20:5n-3	12.5 ± 0.7^{a}	15.8 ± 1.1^{a}	7.4 ± 1.4^{b}
22:3n-3	0.2 ± 0.0	0.2 ± 0.1	0.5 ± 0.3
22:5n-6	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1
22:5n-3	0.3 ± 0.1	0.4 ± 0.1	0.2 ± 0.1
22:6n-3	1.2 ± 0.2^{a}	1.2 ± 0.0^{a}	2.5 ± 0.4^{b}
Total saturates	21.3 ± 0.3	23.1 ± 1.3	22.0 ± 0.8
Total monenes	20.9 ± 1.0^{a}	29.5 ± 2.1^{b}	9.0 ± 0.8^{c}
Total polyenes	24.5 ± 1.1^{a}	31.4 ± 0.7^{b}	20.5 ± 1.0^{c}
Total (n-6) PUFA	6.4 ± 0.3	8.9 ± 0.7	5.4 ± 0.2
Total (n-3) PUFA	18.1 ± 0.9^{a}	22.4 ± 0.9^{b}	15.0 ± 1.2^{c}
n-6 HUFA	2.5 ± 0.1^{a}	3.1 ± 0.1^{b}	1.9 ± 0.1^{a}
n-3 HUFA	14.6 ± 0.7^{a}	18.1 ± 1.1^{b}	11.3 ± 1.2^{c}

Data are means ± s.d. (n=3), and s.d. = 0.0 implies an s.d. <0.05. Totals include some minor components (<0.1%) not shown. HUFA, highly unsaturated fatty acids ($\geq C_{20}$ and with at least 3 double bonds). Values within a row not bearing the same superscript letter are significantly different at $p<0.05$. If no superscript appears, values are not significantly different.

Figure 1. Trypsin-like activity (units larva^{-1}) in Penaeus japonicus larvae and postlarvae feeding on (A) Chaetoceros gracilis during all larval stages plus Artemia nauplii since mysis 1 stage, (B) C. gracilis until mysis 1 and only Artemia nauplii from mysis 1 to postlarva, and (C) only C. gracilis through all larval stages. (n = 5 - 6 for each point). Treatments carrying different superscripts are significantly different ($P<0.05$).

Figure 1.

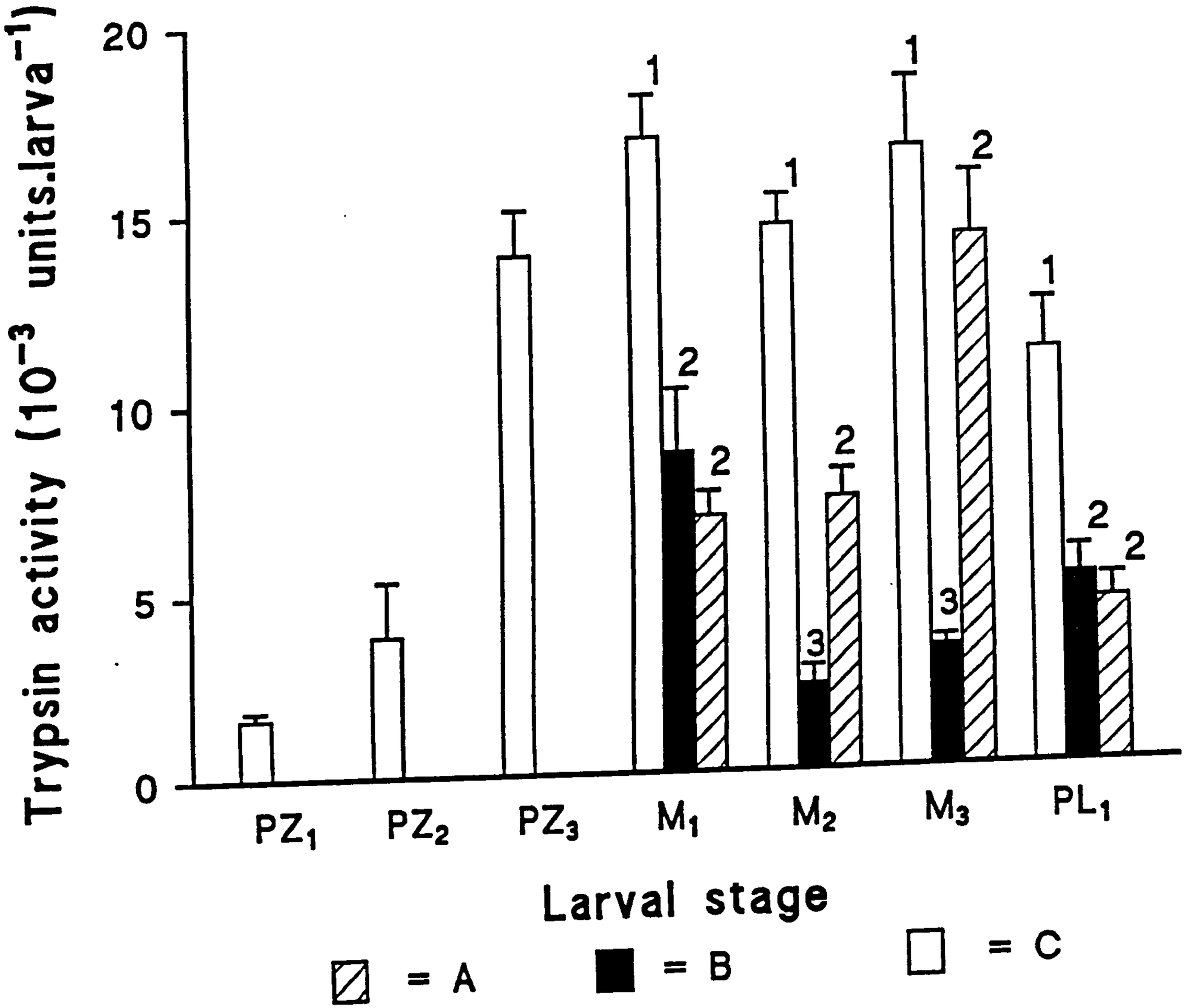

Trypsin activity (10^{-3} units.larva^{-1})
20
15
10
5
0
PZ1
PZ2
PZ3
M1
M2
M3
PL1
Larval stage
= A
= B
= C

Figure 2. Amylase activity (10^{-4} Units $larva^{-1}$) in Penaeus japonicus larvae and postlarvae feeding on (A) Chaetoceros gracilis during all larval stages plus Artemia nauplii since mysis 1 stage, (B) C. gracilis until mysis 1 and only Artemia from mysis 1 to postlarva, and (C) only C. gracilis through all larval stages. (n = 5 - 6 for each point). Treatments carrying different superscripts are significantly different ($P<0.05$).

Figure 3. Amylase:trypsin ratio in Penaeus japonicus larvae and postlarvae feeding on (A) Chaetoceros gracilis during all larval stages plus Artemia nauplii since mysis 1 stage, (B) C. gracilis until mysis 1 and only Artemia nauplii from mysis 1 to postlarva, and (C) only C. gracilis through all larval stages.

Figure 2.

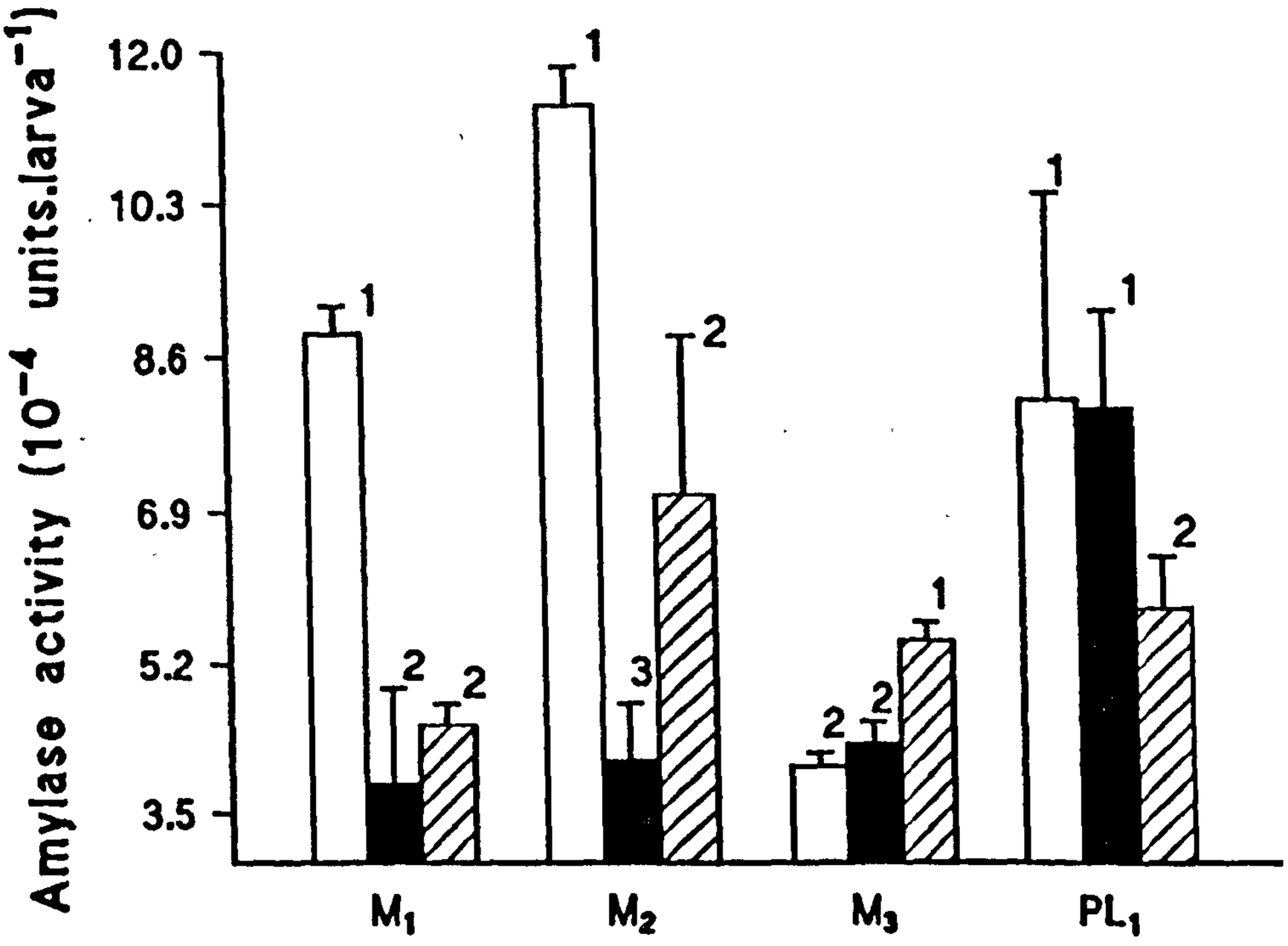
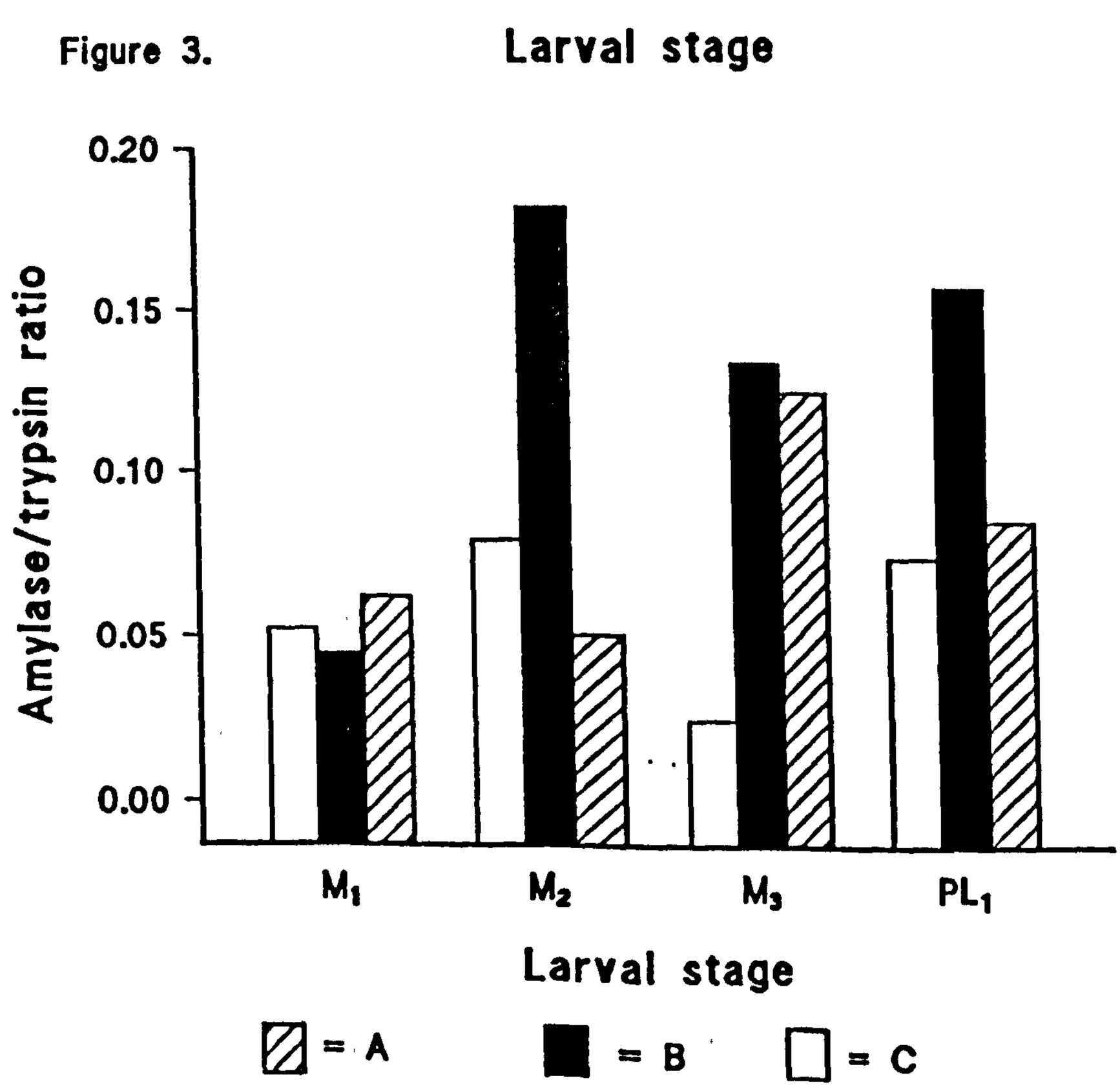


Figure 3.



Chapter 5

DIGESTIVE ENZYME ACTIVITY, GROWTH AND TISSUE COMPOSITION IN PENAEUS JAPONICUS LARVAE FED LIVE AND ARTIFICIAL DIETS.

Parts of this chapter have contributed to the publications;

Le Vay, L., Rodríguez, A., Kamarudin, M.S. and Jones, D.A. (1993) Influence of live and artificial diets on tissue composition and trypsin activity in Penaeus japonicus larvae. Aquaculture 118: 287-297.

Le Vay, L., Rodríguez, A., Mourente, G. and Jones, D.A. (1993) Influence of diet on digestive activity and growth of Penaeus japonicus larvae - implications for nutritional studies. In; Carillo, M., Dahle, L., Morales, J, Sorgeloos, P, Svennevig, N and Wyban, J. (editors) Europ. Aquacult. Soc. Spec. Pub. 19. pp 145.

INTRODUCTION

In the previous chapter it has been shown that dietary levels of protein, and to a lesser extent lipid, required by P. japonicus larvae are much lower than was previously thought. This finding has clear implications for the improvement of artificial diets for penaeid larvae, which usually do not support growth equivalent to that achieved with live foods (see Chapter 1). Although little is known about dietary requirements for protein and lipid by penaeid larvae, most diets tend to mirror the composition of live foods, with protein levels between 30% to 55% (Khannapa 1979, Kurmaly et al. 1989a, Kanazawa 1990, see Chapter 1 Tables 4 and 5). It is possible that effective inclusion levels of protein in artificial diets may reflect the relatively insoluble and indigestible nature of a large portion of that protein resulting from production methods designed to minimise leaching and water pollution (Liao et al. 1988, Amjad et al. 1992).

Growth equivalent to that of larvae reared on live food has been reported using either a low density algal supplement with artificial diets (Amjad et al. 1992, Kamarudin, 1992) or the complete replacement of algae through the protozoeal stages followed by an addition of Artemia during the mysis period (Ottagalli 1991, 1993). Jones et al. (1993b) argue that digestive enzyme reponses to diet are important in determining effectiveness of assimilation of artificial diets by crustacean larvae, and have shown that an algal co-feed can act to stimulate trypsin activity in P. monodon protozoea. Results presented in Chapter 4 have shown that differences in protein content of larval foods may not be reflected in the tissue composition of postlarvae, due to a compensatory response in trypsin activity in mysis larvae. There was also a clear indication of an important role for algal food in achieving optimal growth and survival in larvae fed a mixed diet of zooplankton and phytoplankton, apparently through improved protein digestion and assimilation.

Recent studies have shown an increase in digestive enzyme activity in mysis stage larvae in response to feeding on artificial diets (Kamarudin 1992, Jones et al. 1993b). However, it is not known if these responses are sufficient to significantly improve assimilation of artificial diets. In the present chapter, the experimental approach adopted in Chapter 4 was used to investigate the effect of live and artificial diets on growth, survival, tissue composition and digestive enzyme activity in P. japonicus larvae.

The specific objectives of the study were;

1) To examine the influence of artificial and live feeds on digestive enzyme activity, growth, survival and tissue composition of P. japonicus larvae.

2) To examine the effect of a low density of microalgae fed alongside an artificial

diet on digestive enzyme activity, growth and body composition of P. japonicus larvae.

3) To investigate the potential for manipulation of digestive enzyme responses and growth in protozoeal larvae by incorporation of algal material into an artificial diet.

METHODS

This section describes methods used in a series of experiments carried out at the Instituto de Ciencias Marinas de Andalucia, Cádiz, Spain.

Larval culture

Penaeus japonicus nauplii were supplied from spawnings at the Cupimar S.A. hatchery, San Fernando, Cádiz. Nauplii were maintained in a stock culture of filtered sea water at 27°C until the start of the PZ_1 stage, when they were transferred to experimental treatments. Experimental rearing containers were 25l clear acrylic tubes, 16cm diameter, fitted with conical bases and lowest point aeration. Seawater for all cultures was supplied from a well source, filtered to 1μm and u.v. irradiated. Water renewal was 50% d^{-1}, and 100% when cultures reached M_1. Temperature and salinity during all experiments were maintained at 26-27°C and 32-33°/oo. A photoperiod of 16h:8h light:dark was maintained throughout the experiments. Larvae were stocked at an initial density of $100l^{-1}$.

Chaetoceros gracilis cultures were maintained as described in Chapter 4. Densities of algae remaining in larval cultures after daily water exchange were estimated using a haemocytometer, and sufficient new culture added to maintain the nominal experimental levels of algal cells. Cysts of Artemia were hatched overnight as described in Chapter 4. In treatments receiving Artemia, fresh nauplii were added to larval cultures each day after water exchange.

Frippak artificial diets

Samples of a spray-dried microencapsulated crustacean larval diet (Frippak) was supplied by Sanofi aquaculture. The diet is produced in three size ranges (CAR, CD2 and CD3) which are used sequentially as larvae develop. Particle size distributions, proximate compositions and leaching properties of the diets are presented in Table 1.

Preparation of artificial diets containing phytoplankton

Six 25l carboys were used to culture Rhinomonas reticulata, as described in Chapter 2. Once densities reached approximately 2000 cells μl^{-1}, air input to the cultures was stopped in order to allow settlement overnight. The following day, the clearer upper portion (approximately 50%) of the culture was removed to waste by siphon and the remaining volume concentrated by centrifugation as described in Chapter 2. The cell

density of the pooled concentrate from the 6 cultures was estimated by Coulter counter using diluted samples. Dry weight content of algal material was estimated from cell counts following Kurmaly (1989). The concentrate was frozen to -20^{o}C and transported in a vacuum flask to Heriot-Watt University for inclusion in diets. A frozen slurry of raw ingredients for the Frippak CAR-CD range diet was supplied by Sanofi Aquaculture for spray drying at Heriot-Watt University (by Dr. H. Glass). Two batches of CAR size range diet were produced, one containing 33% Rhinomonas reticulata (HWCAR(R)) and a control diet containing only Frippak slurry, HWCAR). Table 1 shows particle size range, protein content and leaching properties of the two diets.

EXPERIMENT 1; BIOCHEMICAL COMPOSITION AND DIGESTIVE ENZYME ACTIVITY IN PENAEUS JAPONICUS LARVAE FED LIVE AND ARTIFICIAL DIETS

Experimental treatments

Larvae were stocked in 25l experimental containers, as described in Chapter 4, with an initial larval density of 120 l^{-1}. Feeding regimes for the treatments were;

1. Live feeds; C. gracilis (50 - 125 cell μl^{-1}) throughout the larval stages, with the addition of 2-7 Artemia nauplii ml^{-1} during the mysis stages.

2. Artificial diet;
From PZ_1 to PZ_3; Frippak CAR (8mg l^{-1} day^{-1}),
From PZ_3 to M_2; Frippak CD2 (12mg l^{-1} day^{-1}),
From M_2 to PL_1; Frippak CD3 (16mg l^{-1} day^{-1}).

3. Mixed diet; Frippak diet regime as above, plus 10 - 50 cells μl^{-1} C. gracilis through all larval stages.

The artificial diets were hydrated for at least 30 min in distilled water prior to use. Sufficient diet for each day was prepared each morning, stored at 4^{o}C and administered at 08.00h, 14.00h and 24.00h.

Larval sampling

Samples of larvae were examined every day after water was exchanged in the experimental cultures. Larval stages were identified following Hudinaga (1942). Samples were taken the day after the first observation of a particular stage to ensure that larvae were consistently in the middle to late intermoult. Larvae were sorted under binocular microscope and only animals of the required stage selected. In all cases numbers of animals per sample were; 100 PZ_1, 50 PZ_2, 35 PZ_3, 25 M_1, 25

M_2, 20 M_3, 20 PL_1.

Samples from both replicates of each treatment were taken for amylase and trypsin assay, dry weight determination and elemental analysis as decribed in Chapter 4. Samples taken for enzyme assay were quenched in liquid nitrogen and stored at -80^{o}C until assayed.

The length of 20 larvae at each stage were measured using a calibrated graticule in a binocular microscope. Total length was measured from the tip of the rostrum to the end of telson, excluding caudal spines. At the end of the larval period (50% of larvae reaching PL_1), all cultures were harvested and remaining larvae counted. Survival was calculated as a percentage of the original total number stocked in each tube less the number removed during sampling.

Statistical treatment of results

All statistical analyses were carried out using the Minitab (version 7.1) software package. Data were analysed by one-way ANOVA between treatments for each stage and between developmental stages for each treatment. Where appropriate, ANOVA within stages was followed by pairwise comparison of means (Tukey's method for equal sample sizes, Scheffe's for unequal). Where use of ANOVA was not appropriate, the Kruskal-Wallis test was used to rank sample medians. T-tests were used when only two treatments were compared. For all comparison tests, differences are reported as significant at $P<0.05$, except for t-tests where exact probability values are given.

EXPERIMENT 2; GROWTH AND SURVIVAL OF P. JAPONICUS LARVAE FED LIVE AND ARTIFICIAL DIETS.

Experimental treatments

The growth rate and survival of P. japonicus larvae reared using Frippak artificial diets or live feeds (C. gracilis and Artemia nauplii) were compared. The experimental system was as described for Experiment 1, with two tubes assigned to each treatment. Feeding regimes were as described for treatments 1 and 3 in the previous experiment. In addition two experimental diets, HWCAR and HWCAR(R) were tested for influence on trypsin activity, growth and survival of protozoeal larvae. For the latter treatments, replication was constrained by the low amount of diet available and the large size of the culture containers. Thus only single tubes could be allocated to each diet. PZ_1 larvae were stocked at a density of 100 l^{-1} in 25l volume and received diets HWCAR or HWCAR(R) at 8mg l^{-1} day^{-1} from PZ_1-PZ_3. Once cultures had reached 50% M_1, both received Frippak CD2 and CD3 at 16mg l^{-1} day^{-1} until PL_1. One further culture was maintained on the live feed

regime, as described previously. After 24h from first feeding, the latter three cultures were harvested down to 20l, and PZ_1 larvae collected for trypsin assay. Cultures were then maintained at 20l volume until termination of the experiment.

Sampling

Estimation of larval density was routinely carried out, at 9 a.m. each day, by counts of larvae in 10 100ml sample dips. Aeration in each culture was increased for 2-3 min prior to sampling to ensure even mixing of larvae. At 2 p.m. each day, after water exchange, samples were taken from each culture tube for estimation of larval size and stage. The length of 10 larvae from each tube was measured using a calibrated graticule in a binocular microscope. Total length was measured excluding the rostrum in an attempt to eliminate variability introduced by bent rostra. Larval stage of each of the 10 larvae was recorded. At the end of the experiment, samples of PL_1 stage animals were collected from each treatment for length, and dry weight measuremnt. Only mid- to late-stage postlarvae with well developed pleopod setae were selected for sampling.

Statistical treatment of results

Differences in growth within and between treatments were tested by analysis of covariance of length and count data from treatments and replicates nested within treatments against time, using the GLM facility of Minitab. Length data was transformed to natural logarithms to achieve linearity, which was checked by examination of residual plots from regression analysis. Growth during the mysis stages tended to be slower and mortality higher than during the protozoeal stages and hence results from the early and late larval period were analysed separately. Analysis of covariance for the protozoeal period included HWCAR and HWCAR(R) treatments, duplicate Frippak treatment and triplicate live feed treatment. For the mysis period, data from the HWCAR and HWCAR(R) treatments were included as replicates of the Frippak treatment. Where results of covariance analysis showed significant ($p<0.05$) interaction for the replicates term and significant ($p<0.05$) differences between mean slopes within one or more treatments, further analysis used data from replicates cultures without nesting within treatments. Mortality rates were estimated as slopes of regression lines fitted to ln transformed count data (+1 to eliminate zero values).

Differences in trypsin assay data were tested by one-way ANOVA followed by pairwise comparison of means (Scheffé's method). Comparison of postlarval lengths was by one-way ANOVA followed pairwise comparisons (Tukey's method).

RESULTS

EXPERIMENT 1; BIOCHEMICAL COMPOSITION AND DIGESTIVE ENZYME ACTIVITY IN PENAEUS JAPONICUS LARVAE FED LIVE AND ARTIFICIAL DIETS

Nutrient composition of live and artificial feeds

Proximate composition, leaching properties and particle size distribution of the artificial diets are presented in Table 1. The protein content (53.8%) of Frippak CAR reduced after 1h leaching to 46.6%. As overall dry weight loss was 32%, these changes indicate percentage leach loss of 31% for protein. In the case of the larger size range CD2 and CD3 diets, protein leach losses were lower (26% and 25%), resulting in protein contents of 51.5% and 56.7% respectively. Proximate compositions of the live feeds C. gracilis and Artemia nauplii are presented in Chapter 4.

Size and survival of larvae fed live and artificial diets.

Dry weight and length data for all larval stages for each treatment are presented in Figures 1a and 1b. Survivorship and time taken to reach PL_1 are presented in Table 2. Despite PZ_1 and PZ_2 larvae fed the artificial diet being significantly smaller in terms of dry weight and length than those feeding on the alga, there was no significant difference in length or dry weight of PZ_3 larvae from all treatments. At all three protozoeal stages, larvae feeding on the Frippak diet plus C. gracilis were not significantly different in size to those feeding on the alga alone. Except for lengths at M_3, there were no significant differences dry weights and lengths of M_1 to PL_1 larvae fed live food or artificial diet plus C.gracilis, while mysis larvae and postlarvae fed the artificial diet were significantly smaller than those fed live food ($P<0.05$).

In all cultures fed C. gracilis or artificial diet plus C. gracilis more than 50% occurrence of M_1 larvae was observed after 5 days, while those receiving only the artificial diet did not reach this stage of development until one day later. In cultures reared on artificial diet 50% occurrence of PL_1 stage animals was observed after 10.5 days, compared to 9 days for cultures receiving live feeds and artificial diet plus C. gracilis. Mean survival to PL_1 was 78.0% in the live food treatment, 39.2% in the artificial diet treatment and 79.5% for larvae fed the artificial diet plus C. gracilis.

Elemental composition of larvae

Results of elemental analyses are shown in Figures 2a and 2b. The pattern of change in nitrogen and carbon content of larvae fed live food or artificial diet plus C. gracilis

through development was characterised by significant increases during the protozoea and first mysis stages ($p<0.001$). After an anomalous drop at M_2, the higher levels were maintained at M_3 and PL_1. In contrast there was no significant change in nitrogen or carbon content during the development of larvae fed the artificial diet alone. There were some significant differences between nitrogen and carbon contents of larvae from the three treatments. At PZ_1, the nitrogen and carbon contents of larvae fed live food or artificial diet plus C. gracilis were significantly lower than those of larvae fed artificial diet only. By M_1 the nitrogen content of larvae fed the live food or artificial diet plus C. gracilis had risen to levels significantly higher that of larvae fed artificial diet ($p<0.05$). Postlarvae reared on live food or artificial diet plus C. gracilis contained significantly higher levels of nitrogen and carbon than those reared on the artificial diet alone ($p<0.05$).

Digestive enzyme activity in larvae fed live and artificial diets.

Figure 3 shows tissue trypsin activity ($x10^{-4}$ units μg^{-1}) for samples of larvae from the three feeding regimes. Trypsin activity in PZ_1 larvae feeding on C. gracilis was significantly higher than that in larvae feeding on Frippak CAR, but not significantly different from that in larvae feeding on Frippak plus C. gracilis. At PZ_2 and PZ_3, trypsin activity in larvae feeding on C. gracilis was significantly higher than that of larvae from either of the other treatments. There was no significant difference between trypsin activity in PZ_3 larvae feeding on CAR or the artificial diet plus C. gracilis. This pattern persisted at M_1. At M_2 the trypsin activity in larvae feeding on Frippak CD3 was significantly lower than that recorded for larvae feeding on CD3 plus C. gracilis. Similarly high values were recorded for these treatments at M_3, but trypsin activity in the live food treatment dropped markedly at this stage. The low trypsin activity in the live food treatment persisted at PL_1 while values for postlarvae feeding on CD3 remained significantly higher, but only about half the levels recorded for the previous stage. In postlarvae feeding on CD3 plus C. gracilis, trypsin activity did not drop from the high values observed in the late mysis stages.

Figure 4 shows tissue amylase activity ($x10^{-5}$ units μg^{-1}) for samples of larvae from the three feeding regimes. Amylase activity in PZ_1 larvae feeding on CAR was significantly higher than for larvae feeding on either C. gracilis or the mixed diet. At PZ_2, amylase activities in larvae feeding on CAR and CAR plus C. gracilis were similar, while these values were over three times higher than activity in larvae feeding on C. gracilis alone. At PZ_3 the amylase activity in larvae feeding on the alga had risen to the same level as in larvae feeding on CAR or CAR plus C. gracilis. For all three treatments amylase activity rose markedly at M_1. High levels were maintained at M_2 in larvae feeding on CD3 and CD3 plus C. gracilis with no

data recorded for the live feed treatment. At M_3 amylase activity in larvae feeding on live feed and CD3 dropped, with no data recorded for CD3 plus alga. Amylase activity recorded in postlarvae feeding on live food was significantly higher than that recorded in postlarvae feeding on the artificial diet while values for animals feeding on CD3 plus C. gracilis were significantly lower again.

Effect of the inclusion of Rhinomonas reticulata in an artificial diet on trypsin activity in PZ_1 larvae.

Trypsin activities in samples of larvae feeding on C. gracilis , HWCAR and HWCAR(R) are shown in Table 3. Highest activity (20.9 $\pm$ 3.1 units $larva^{-1}$) was recorded in larvae feeding on C. gracilis. However the low activity found in larvae feeding on HWCAR (6.5 $\pm$ 1.6 units $larva^{-1}$) was significantly enhanced by the inclusion of Rhinomonas reticulata (10.2 $\pm$ 2.5 units $larva^{-1}$).

EXPERIMENT 2; GROWTH AND SURVIVAL OF P. JAPONICUS LARVAE FED LIVE AND ARTIFICIAL DIETS.

Growth

Growth rates (ln mm day^{-1}) of protozoeal (days 1 to 5) and mysis (days 5 to 8) larvae are shown in Tables 4 and 5. As preliminary covariance analysis showed significant differences within treatments, these tables show analysis using separated replicates. The statistical analysis compares growth rate for each treatment to the overall growth rate for data from all treatments. For protozoeal larvae the overall average growth rate was 0.242 $\pm$ 0.003 ln mm day^{-1}, and there were significant differences in growth rates between cultures. Both replicates of the treatment receiving Frippak CAR exhibited significantly better than average growth ($p = 0.017$ and 0.039) while two of the live feed replicates exhibited significantly poorer than average growth ($p = <0.001$ and 0.001). However, in the third live feed replicate, growth was not significantly different from the overall average ($p = 0.335$). The HWCAR and HWCAR(R) treatments produced very similar growth rates which were not significantly different from the overall average ($p = 0.055$ and 0.380).

During the mysis stages the overall growth rate for all treatments was lower (0.073 $\pm$ 0.003 ln mm day^{-1}) than for protozoeal larvae (Table 5). Of the four cultures receiving Frippak CD2-CD3, three produced growth rates which were not significantly different from the overall rate ($p = 0.683$, 0.564 and 0.202), while the fourth produced significantly poorer ($p = 0.041$). There was greater variability in growth rates from the three live food cultures. The growth rate for the first replicate was significantly lower than the overall rate ($p = 0.001$) while that of the second was highly significantly better ($p < 0.001$). The growth rate of the third live feed

replicate was intermediate and not significantly different from the overall rate (p = 0.226).

Dry weight and length of samples of postlarvae are shown in Table 6. Mean dry weights of postlarvae from the cultures which had been reared exclusively on the Frippak diets exclusively (60.1μg - 62.4μg) were similar to those recorded for the first experiment (Figure 1). In two cases, mean dry weights of postlarvae reared on live food were consistent with those recorded in the first experiment (74.0 μg and 83.4μg) while the third replicate produced smaller postlarvae (60.9μg). The final weights of postlarvae in treatments which initially had received the HWCAR and HWCAR(R) diets were substantially smaller than those from all other treatments (43.3 and 48.1μg).

Survival

Mortality rates (ln larvae $100ml^{-1}$ day^{-1}) for the protozoeal stages (days 1 to 5) are shown in Table 7 and those for mysis stages (days 5 to 8) in Table 8. During the protozoeal stages, significant mortality was recorded in only one replicate of the Frippak treatment (p = 0.044), but not for the second (p = 0.468). In two replicates from the live feed treatment mortality rates were highly significant (0.002 and <0.001), while in the third replicate no significant mortality was recorded (p = 0.072). Neither of the treatments receiving HWCAR or HWCAR(R) exhibited significant mortality rates (p = 0.128 and 0.487).

During the mysis stages only one replicate from of the Frippak CD2-CD3 treatment showed significant mortality (p = 0.047), while two of the three live feed replicates showed highly significant losses (p = 0.001 and <0.001). Mean survival for the four cultures receiving artificial diets was 78.5% at D5 and 64.3% at D8, while mean survival for the three cultures receiving live feeds was 49% at D5 and 27.3% at D8.

DISCUSSION

Diet composition and nutritional requirements of penaeid larvae

Table 5 (Chapter 1) summarises the available literature on nutritional requirements of penaeid larvae. From Chapter 4, it is apparent that although data in the literature may represent dietary compositions which have allowed successful larval culture, optimal percentage levels of protein, lipid and carbohydrate remain to be defined. Little is known about the importance of digestibility of different proteins or the effect of varying inclusion levels in diets. Leach loss of nutrients from artificial diets before ingestion are not commonly taken into account in studies of penaeid larval nutrition, suggesting that nutritional requirements for some dietary components may be

overestimated. The rapid leach-loss of the most soluble, and presumably the most easily assimilated, protein suggests that actual protein ingested may differ both quantitatively and qualitatively from that included in the diet formulation.

Growth and survival of P. japonicus larvae fed live and artificial diets.

Hudinaga (1942) reports the results of growth trials with P. japonicus larvae from an extensive series of spawnings, reared using diatoms and Artemia nauplii. Comparison with larval length data in Figure 1 shows that the live feed treatment and the treatment receiving Frippak diets plus C. gracilis produced protozoeal larvae within the range of sizes reported by Hudinaga (1942). M_1 and M_2 larvae from these treatments were slightly larger than the maximum reported by Hudinaga, while M_3 larvae were smaller than his average value and PL_1 from both these treatments were slightly smaller than his minimum recorded length. In contrast, lengths of larvae fed only the Frippak diets fell within the range reported by Hudinaga from PZ_1 to M_1 but were below his minimum values at M_1 and PL_1. Thus results from the first experiment indicate that while the size of larvae from the live feed treatment was comparable with values from the literature for P. japonicus, the Frippak CAR diet was only able to produce larvae of equivalent size at PZ_3. Despite comparable size, it should be noted that the duration of the protozoeal stages was longer for larvae feeding on the artificial diet, indicating a slower growth rate in this treatment, which was improved to equivalence with live feeds by the algal co-feed.

In the second experiment, where data ~~was~~ were analysed by comparison of slopes of linear regression fits to length and count data (Tables 4,5,7 and 8), it is impossible to differentiate between growth and survival of larvae receiving live or artificial foods because of the great variability between replicates in the live food treatment. However, comparison of data shown in Figure 1 and Table 6 of the present chapter and Table 2 of chapter 4, shows that there was also considerable variation in weight of postlarvae from the different spawnings reared on the same mixed live food treatment (C. gracilis and Artemia). However, final weights of postlarvae reared on the Frippak artificial diets in the two experiments in the present study were fairly consistent (Figure 1, Table 6), especially when the HWCAR and HWCAR(R) treatments are excluded. In both cases better growth on live food is indicated - except in one relicate from the second experiment.

Digestive enzyme activity in larvae feeding on live and artificial diets.

Trypsin activity represents 40% to 60% of proteolytic activity of P. japonicus (Galgani el al 1984, 1985, Tsai et al. 1986). In this and other penaeid species trypsin is the dominant component of the digestive enzyme complement during larval development (Lovett and Felder, 1989, Kamarudin 1992, Jones et al.. 1993a,b). In

general, trypsin activity in penaeid larvae receiving live food has been found to peak at PZ_3 to M_1, declining towards PL_1 and this pattern has been observed in P. japonicus larvae by Laubier-Bonichon et al. (1977). Galgani and Benyamin (1985) found maximum trypsin activity at PZ_2, but did not present results for the following two stages. A similar pattern has been observed in live feed treatments in the present study and in Chapter 4. In the present study, tissue trypsin activity peaked at PZ_3 for larvae feeding on C. gracilis and declined through the mysis stages. Kamarudin (1992) reported a peak in trypsin activity one stage later at M_1 in larvae receiving a similar live food treatment, with a less marked decline in activity towards PL_1. However, the overall pattern of change of trypsin activity with development was similar.

The pattern of trypsin activity observed in P. japonicus larvae feeding on artificial diets is similar to results reported by Kamarudin (1992) for the same species, Jones et al. (1993) for P. monodon and Le Moullac et al. (1993) for P. vannamei. In the early larval stages there is a relatively poor trypsin response to artificial diets. Once the mysis stages are reached, the ability to respond to artificial diets with elevated secretion of trypsin is developed.

The peak in amylase activity at M_1 to M_2 observed in the live feed treatment in the present study agrees with the pattern of activity for this enzyme reported by Laubier-Bonichon (1977) and Kamarudin (1992). In the present study, feeding on artificial diets produced elevated amylase levels in PZ_1 and PZ_2 larvae, compared to those feeding on C. gracilis, but this influence did not persist further through larval development. In contrast, Kamarudin (1992) found elevated amylase levels in larvae feeding on artificial diets through all larval stages except M_3. In his study, amylase activity at PL_1 was higher in larvae feeding on artificial diets than live feeds, while the reverse was observed in the present study.

Values for trypsin activity in PZ_1 and PZ_2 larvae from the the present study were similar to those reported by Kamarudin (1992). However tissue trypsin activity in larvae feeding on Frippak diets from PZ_3 to M_3 were higher in the present study than found by Kamarudin (1992). Similarly, tissue amylase activity in all larval stages feeding on Frippak diets were found to be higher in the present study than recorded by Kamarudin (1992). As both Kamarudin's and the present results for P. japonicus were from different spawnings, reared in the same laboratory using similar diets, it is apparent that considerable variation in digestive activity between spawnings is to be expected in terms of absolute levels of activity as well as relative responses to different dietary treatments. This is supported by the relatively high trypsin activities recorded in PZ_1 larvae feeding on C. gracilis, HWCAR and HWCAR(R) in the second experiment of the present study (Table 3).

Results from the present study and Chapter 4 demonstrate flexibility in enzyme response to diet during both the protozoeal and the mysis stages. Samain et al. (1981) propose a model in which dietary assimilation by Artemia is related to the product of digestive enzyme activity and ingestion rate. Jones (1988) found that in PZ_1 P. monodon larvae, the rate of ingestion of nutritionally inert particles such as carbon powder was higher than that observed with algal food (Tetraselmis chuii). The rate of ingestion of an microencapsulated artificial diet was also higher than that of the live food. Such flexibility in ingestion rates would allow P. japonicus larvae to process more of an algal food of low nutritional value, as used in the present study, and hence maximise nutrient assimilation. Following the same argument, protozoeal penaeid larvae can maximise the uptake of nutrients from relatively indigestible artificial diets, and hence support growth and survival, by increasing the amount of food processed. However, the relatively low trypsin response to the artificial diet would act to limit assimilation, with consequent low growth rates.

In the present study, PZ_1 larvae which were fed the Frippak artificial diet plus a low density of C. gracilis exhibited trypsin activity which was significantly higher than that in larvae fed the artificial diet alone. A similar effect has been reported for P. monodon (Jones et al. 1993a) who have suggested that stimulation of trypsin activity by some component of the algal cells might explain the enhancement of growth observed with an algal co-feed. The algal food may also provide a supplement of more digestible nutrients, though this contribution would be small due to its low nutrient content.

In the first experiment of present study growth during the first two protozoeal stages was enhanced in the treatment receiving artificial diet plus algal food. Successful growth during these stages, which represent the period of highest percentage growth in penaeid larvae (Hudinaga 1942, Kurmaly et al. 1989b), may be critical in determining the subsequent rate of development of penaeid larvae. Recently Fletcher et al. (1994) have found that an algal supplement fed during the PZ_1 stage only, in addition to a nematode diet, can improve growth and survival of Penaeus indicus during later development. If nutrition at PZ_1 does influence later growth and development, then stimulation of trypsin activity at this stage by an algal co-feed may be of great importance. However, the effects of early nutritional deficiencies on later development of penaeid larvae is an interesting area which as yet remains to be fully investigated.

The high levels of trypsin activity recorded in larvae feeding on C. gracilis in Chapter 4 and in the present study may due to be a general response to low dietary protein availability combined with a direct stimulation of secretion by an active

component of the alga. The higher trypsin response in PZ_1 larvae feeding on the HWCAR(R) diet compared to the HWCAR control diet demonstrates that it is possible to manipulate digestive enzyme responses in penaeid larvae by inclusion of algal additives to diet formulations. It is interesting that these diets were virtually identical in total protein content and protein leach rates, suggesting that PZ_1 larvae respond to an active stimulatory component of the alga as well as to the overall protein content of the diet. That the effect was observed despite the high leaching properties of these diets suggests that any active component is unlikely to be readily water soluble. Taken together with the fact that the effect of the algal additive has survived the processes of freezing and spray drying present results suggest that future characterisation and identification of stimulatory algal components might be achieved using spray-dried diets as the medium for delivery of isolated fractions.

In Chapter 4, high trypsin activity in mysis larvae feeding on C. gracilis has been interpreted as a response, at least in part, to the low protein content of the alga. A response of high digestive enzyme secretion to substrate scarcity has been suggested for Calanus helgolandicus (Harris et al. 1986). Samain et al. (1985) observed high levels of amylase activity in Artemia in response to starch-free diets. A similar suggestion for Calanus pacificus also supports the idea that high enzyme secretion may be a response to low food availability (Hasset and Landry 1983). It appears that once P. japonicus larvae reach the mysis stages they are well adapted to respond to diet composition with appropriate enzyme secretion and hence that the high trypsin response to the Frippak diets is a result of the low availability of readily digestible protein in the diet, particularly given the leach loss of readily soluble protein components. This would also explain the rapid drop in trypsin activity once larvae start to feed on Artemia nauplii, which would provide an easily assimilated mixture of soluble proteins, peptides and amino-acids (Fyhn, 1989). Lan and Pan (1993) have demonstrated a more effective in vitro digestion of Artemia nauplii by an extract of P. monodon hepatopancreas compared to that of a range of protein ingredients to formulated diets. However, the observation that an algal co-feed can elevate trypsin activity in mysis larvae to levels above those produced in reponse to an artificial diet alone is evidence that two mechanisms of control of enzyme secretion may operate in mysis stage larvae.

Elemental composition of larval stages of P. japonicus reared on live and artificial diets

Present results for carbon and nitrogen content of P. japonicus larvae are within the range of values reported elsewhere for a wide range of wild tropical and sub-tropical zooplankton species (Ikeda 1974) as well as for cultured larvae of several decapod

species (Childress and Nygaard 1974, Dawirs 1980, Anger and Jacobi 1985, Anger and Püschel 1986). Tobias-Quinitio and Villegas (1982) report protein contents of 35.7% for P. monodon PZ_1 larvae feeding on Chaetoceros calcitrans (protein content 23.9%) and 41.7% for larvae feeding on Tetraselmis chuii (protein content 49.2%). In the same study, protein content of M_3 P. monodon reared on live food was reported to be in the range 49.7% to 51.1%. Their results for PZ_1 larvae support the conclusion from the present study that diet composition can be reflected in larval composition even within the first feeding stage. PZ_1 P. japonicus larvae from the present study have a higher organic content (in terms of C,N) when feeding on CAR than on either C. gracilis alone or a mixture of CAR and C. gracilis. However, in a variety of decapod larvae it has been shown that organic content is highest post-moult, decreasing during the intermoult period and rising again at premoult (Anger 1988 and references therein). While care was taken to sample at the mid-point of each intermoult, PZ_1 larvae were sampled from all treatments at the same time. It may be be that, due to the slower rate of development of larvae in the artificial diet treatment, these were sampled at an earlier stage of the PZ_1 intermoult period than larvae from the other treatments, accounting for the relatively high C and N content at this stage.

In protozoeal larvae fed C. gracilis alone, the very low energy content of the alga would have necessitated efficient assimilation, achieved with a high digestive enzyme response and possibly an increase ingestion rate (Harris et al. 1986, Jones 1988). Significant increases in nitrogen and carbon content during the protozoeal stages in the live food treatment presumably reflect an increased ability to assimilate dietary nutrients, coinciding with the development of the AMD (Abubakr 1991, Abubakr and Jones 1992).

Once larvae begin to feed on Artemia nauplii, continued high growth and retention of high levels of carbon and nitrogen is possible with lower trypsin activity. Despite the high levels of trypsin activity observed, digestion of the artificial diet was insufficient to support optimal growth or carbon and nitrogen retention. Weight specific ingestion rates by mysis larvae are substantially lower than in protozoea (circa 140% body weight day^{-1} compared to 500% day^{-1}, Kurmaly et al. 1989b). Although growth rates are also lower during the mysis stages, the amount of artificial diet processed may become the factor which limits assimilation and growth at this stage of development. The supplementation of the artificial diet with a low density of algal cells appears to provide sufficient extra digestible nutrients to support growth equivalent to that observed in larvae reared on live feeds. Alternatively, given the low nutritional value of the alga, it is possible that the provision some algal component, lacking in the artificial diet, contributed to the

beneficial influence of the algal co-feed.

Table 1. Proximate composition, leaching properties and size distribution of Frippak artificial diets CAR, CD2, CD3 and of diets HWCAR and HWCAR(R) produced at Heriot-Watt University

a) Proximate composition (% of dry weight)

Diet	Lipid	Protein	Ash	Carbohydrate/fibre
CAR	16.6	53.8	16.3	13.3
CD2	21.5	52.6	15.0	11.3
CD3	20.5	52.9	11.9	14.7
HW CAR	n.d.	53.0	n.d.	n.d.
HW CAR(R)	n.d.	47.4	n.d.	n.d.

c) Percentage leach loss of dietary components

	Dry weight	Protein
CAR	32	31
CD2	24	26
CD3	30	25
HWCAR	n.d.	52
HWCAR(R)	n.d.	47

d) Size distribution of diets (μm)

	Minimum	Maximum	Mode	Median
CAR	1.2	247	28	25
CD2	1.2	300	40	32
CD3	1.2	493	150	137
HW CAR	1.2	224	17	16
HW CAR(R)	1.2	224	19	17

Data supplied by Sanofi Aquaculture and Dr. J.R. Stark.

n.d.= no data

Table 2. Penaeus japonicus. Duration of larval development from PZ_1 to M_1 and from PZ_1 to PL_1, and survival(%) at PL_1 for treatments; (A) C. gracilis from PZ_1 to M_1, Artemia nauplii plus C. gracilis from M_1 to PL_1 (B) Frippak artificial diet from PZ_1 to PL_1 (C) Frippak artificial diet plus C. gracilis from PZ_1 to PL_1. Values are means from two replicates for each treatment.

Treatment	Survival to PL_1	Duration to M_1	Duration to PL_1
Live food	79.8%	5	9
Frippak	43.8%	6	10.5
Frippak + alga	79.5%	5	9

Table 3. Trypsin activities for samples of PZ_1 stage Penaeus japonicus larvae after 24H feeding on diets HWCAR, HWCAR(R) and C. gracilis.

Diet	Trypsin activity ($\times 10^{-4}$ Units $larva^{-1}$)
C. gracilis	20.9 ± 3.1 (n=7)
HWCAR	10.2 ± 2.5 (n=9)
HWCAR(R)	6.5 ± 1.6 (n=7)

All treatments significantly different at $p<0.05$, Scheffé's method pairwise comparison.

Table 4. Penaeus japonicus. Growth rates (ln mm day^{-1}) of larvae from day 1 to day 5 fed Frippak CAR, live feed (C. gracilis) and diets HWCAR and HWCAR(R). Differences from average rate for all treatments have been tested for significance by analysis of co-variance (table in Appendix 5)

Diet	Replicate	Growth rate	Difference from average rate	s.d. of difference	t-value	P
CAR	1	0.260	0.018	0.0075	2.41	0.017
	2	0.257	0.015	0.0073	2.07	0.039
Live feed	1	0.249	0.007	0.0073	0.97	0.335
	2	0.204	-0.037	0.0074	5.06	<0.001
	3	0.223	-0.019	0.0055	3.33	0.001
HW CAR	1	0.252	0.011	0.0056	1.92	0.055
HW CAR(R)	1	0.246	0.005	0.0056	0.84	0.380

Table 5. Penaeus japonicus. Growth rates (ln mm day^{-1}) from day 5 to day 8 of larvae fed Frippak artificial diets and live feed (Chaetoceros gracilis and Artemia nauplii). Differences from average rate for all treatments have been tested for significance by analysis of co-variance (table in Appendix 5).

Diet	Replicate	Growth rate slope	Difference from average slope	s.d. of difference	t-value	P
Frippak	1	0.069	-0.004	0.0091	-0.41	0.683
	2	0.067	-0.005	0.0093	-0.58	0.564
	3	0.081	0.009	0.0068	1.28	0.202
	4	0.059	-0.014	0.0068	-2.02	0.041
Live feed	1	0.044	-0.029	0.0090	-3.26	0.001
	2	0.108	0.035	0.0090	3.92	<0.001
	3	0.081	0.008	0.0067	1.21	0.226

Table 6. Penaeus japonicus. Length (mm) and dry weight (μg) of stage 1 postlarvae reared on a) Frippak CAR-CD2-CD3 b) HWCAR or HWCAR(R) followed by CD2-CD3 and c) live feed (C. gracilis and Artemia nauplii). All treatments measured on D9, Values are mean values ± s.d. (n=20 for length, n=3-5 for weight). For length data, treatments not bearing the same superscript are significantly different at $p<0.05$ (Tukey's pairwise comparison).

Treatment	Replicate	Length ± s.d. (mm)	Dry weight (μg)	Days to reach 50% PL_1
Live feed	2	4.04 ± 0.16^{a}	83.4 ± 5.6	9
Live feed	3	3.87 ± 0.11^{b}	74.0 ± 6.1	8
Frippak	2	3.76 ± 0.10^{bc}	60.1 ± 1.4	8
Frippak	1	3.74 ± 0.15^{bc}	62.4 ± 3.9	8
Live feed	1	3.72 ± 0.12^{c}	60.9 ± 4.3	8
HWCAR+Frippak	1	3.51 ± 0.16^{d}	48.1 ± 8.3	8
HWCAR(R)+Frippak	1	3.48 ± 0.19^{d}	43.6 ± 4.3	8

n.d. = no data

Table 7. Penaeus japonicus. Mortality (ln larvae $100ml^{-1}$ day^{-1}) estimated as slopes of regression lines fitted to ln larval count (+1) from D1 to D5 for treatments feeding on Frippak CAR, live feed (C. gracilis) and diets HWCAR and HWCAR(R) (tables in Apppendix 5).

Diet	Replicate	Mortality (slope)	F value	d.f.	P
CAR	1	-0.042	4.35	1,36	0.044
	2	-0.026	0.54	1,37	0.468
Live feed	1	-0.068	3.43	1,37	0.072
	2	-0.120	11.08	1,37	0.002
	3	-0.147	18.15	1,35	<0.001
HWCAR	1	-0.047	2.41	1,38	0.128
HWCAR(R)	2	0.015	0.49	1,36	0.487

Table 8. Penaeus japonicus. Mortality (ln larvae $100ml^{-1}$ day^{-1}) estimated as slopes of regression lines fitted regression to ln larval count (+1) D5 to D8 for treatments feeding on Frippak CAR, live feed (Artemia nauplii and C. gracilis). Tables in Appendix 5.

Diet	Replicate	Mortality (slope)	F value	d.f.	P
Frippak	1	-0.0660	3.57	1,38	0.660
	2	-0.0664	4.23	1,34	0.047
	3	-0.0691	3.13	1,38	0.085
	4	-0.0226	0.40	1,36	0.531
Live feed	1	-0.1709	13.87	1,36	0.001
	2	-0.3270	42.31	1,35	<0.001
	3	0.0524	1.26	1,35	0.269

Figure 1. **(a)** Dry weight (μg) and **(b)** length of P. japonicus larvae from cultures reared using the following feeding regimes;

1. Live feeds; C. gracilis (50 - 125 cell μl^{-1}) throughout the larval stages, with the addition of 2-7 Artemia nauplii ml^{-1} during the mysis stages.

2. Artificial diet; PZ_1 to PZ_3 Frippak CAR (8mg l^{-1} day^{-1}), PZ_3 to M_2 Frippak CD2 (12mg l^{-1} day^{-1}), M_2 to PL_1 Frippak CD3 (16mg l^{-1} day^{-1}).

3. Mixed diet; Frippak diet regime as above, plus 10-50 cells μl^{-1} Chaetoceros gracilis through all larval stages.

Values are means $\pm$ s.d. For weights n = 3 - 4 for each value, for lengths n = 20 for each value. Within each larval stage treatment means marked with different superscripts are signficantly different ($p<0.05$). Data in Appendix 5.

Figure 1a.

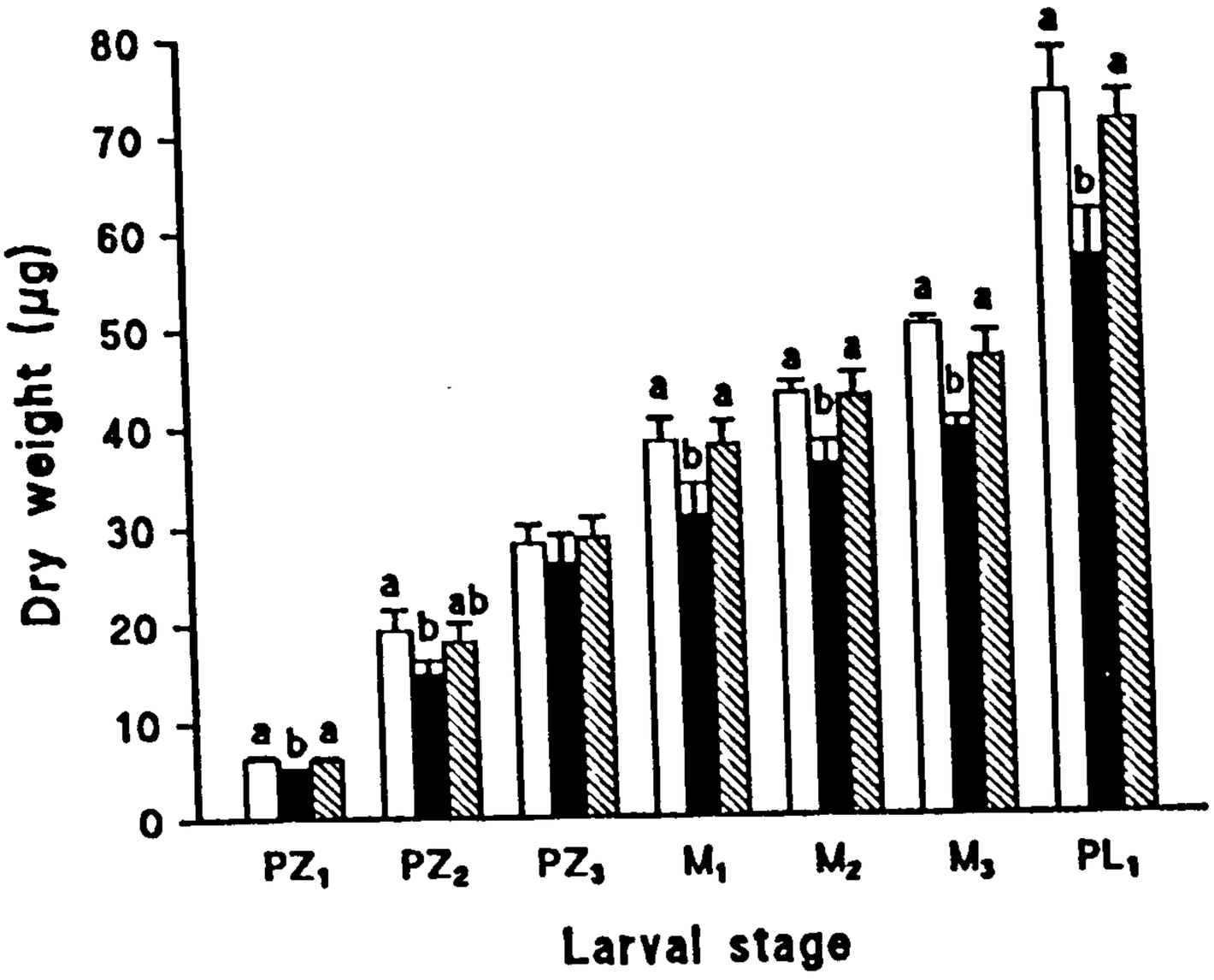


Figure 1b.

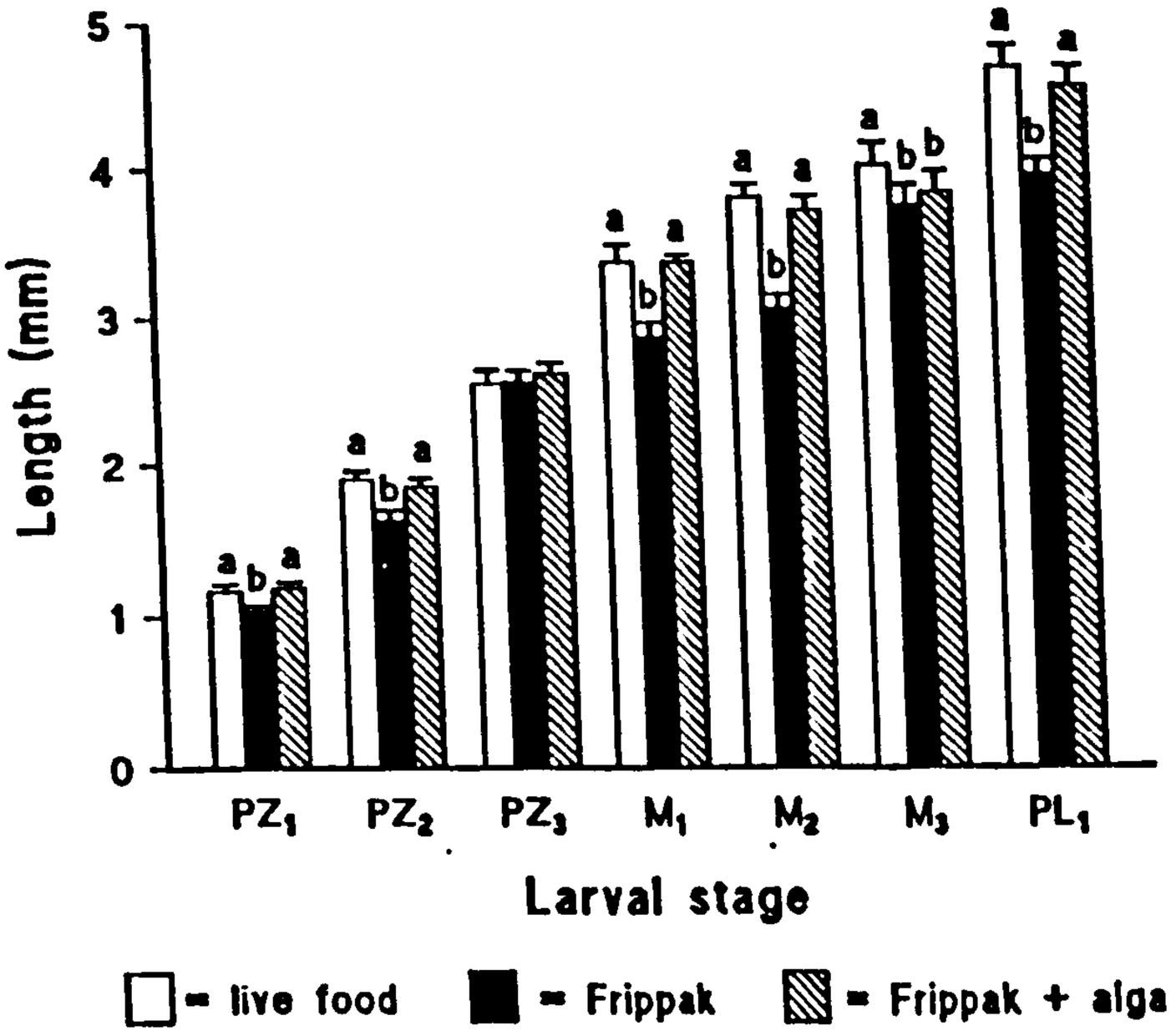


Figure 2. (a) Nitrogen content and **(b)** carbon content (% of dry weight) of Penaeus japonicus larvae reared using the following feeding regimes;

1. Live feeds; C. gracilis (50 - 125 cell μl^{-1}) throughout the larval stages, with the addition of 2-7 Artemia nauplii ml^{-1} during the mysis stages.
2. Artificial diet; PZ_1 to PZ_3 Frippak CAR (8mg l^{-1} day^{-1}), PZ_3 to M_2 Frippak CD2 (12mg l^{-1} day^{-1}), M_2 to PL_1 Frippak CD3 (16mg l^{-1} day^{-1}).
3. Mixed diet; Frippak diet regime as above, plus 10-50 cells μl^{-1} Chaetoceros gracilis through all larval stages.

Values are means ± s.d., n = 3-5. Within each larval stage treatment means marked with different superscripts are signficantly different ($p<0.05$). Data in Appendix 5.

Figure 2a

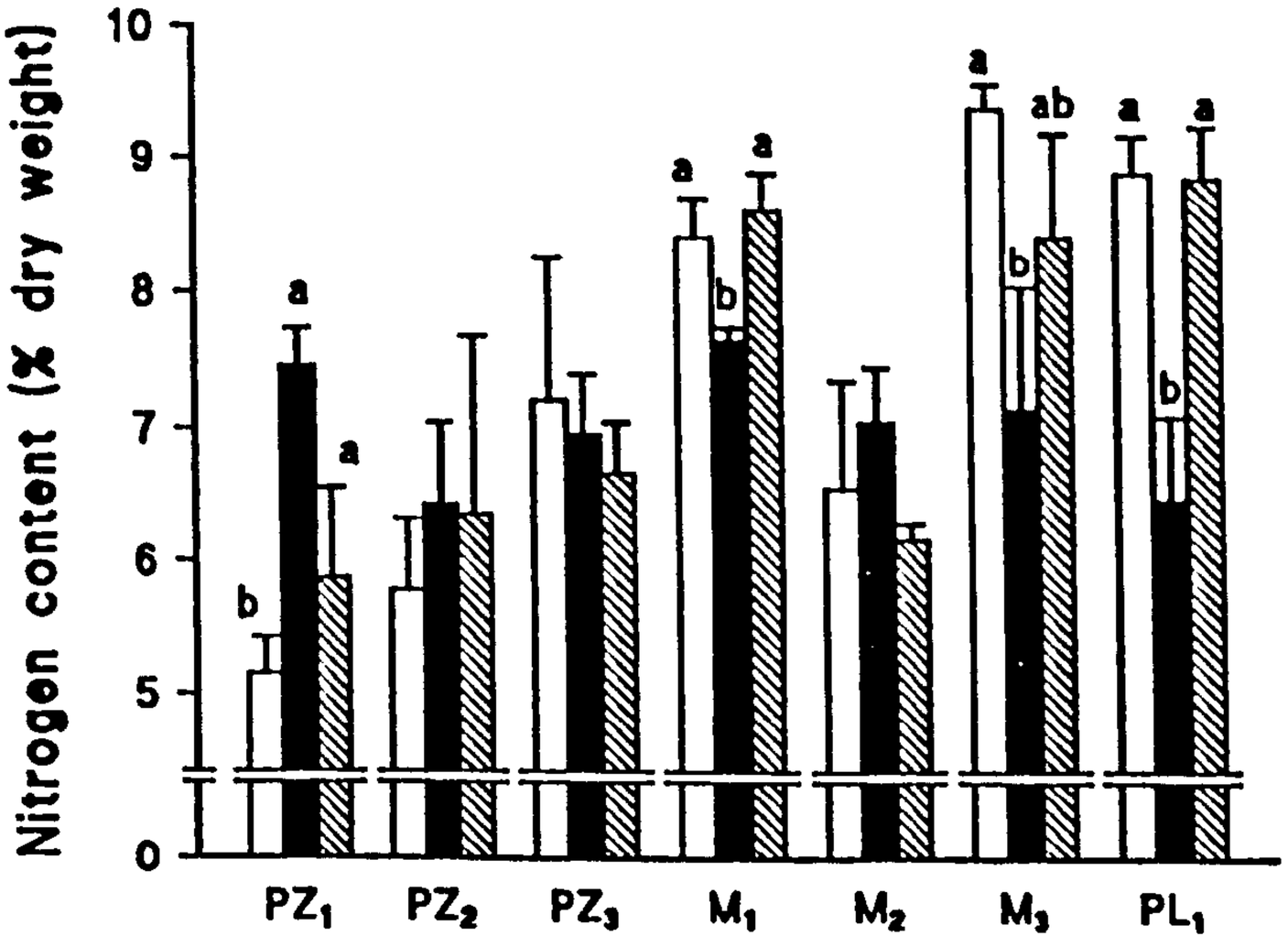


Figure 2b.

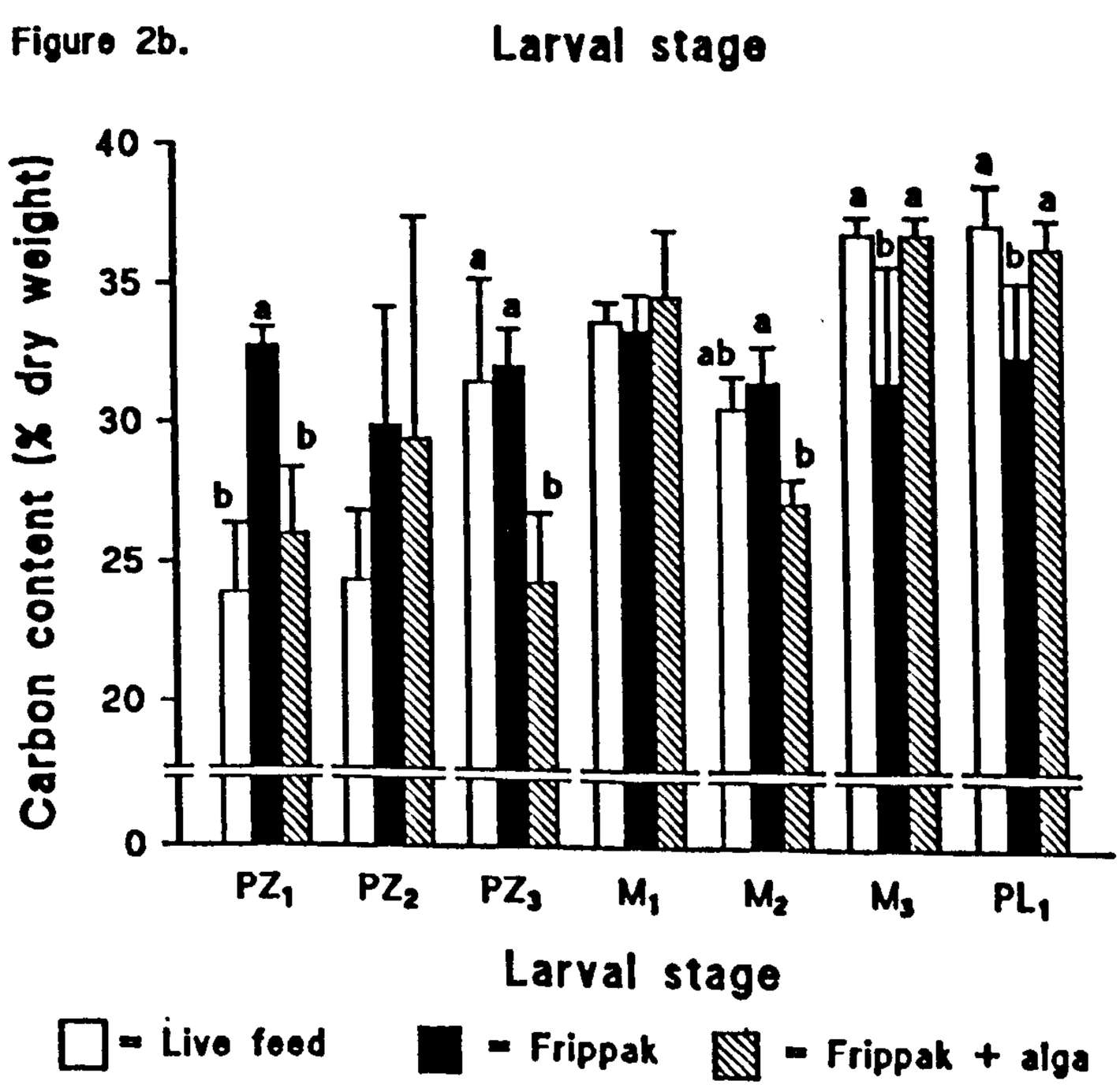


Figure 3. Tissue trypsin activity ($x10^{-4}$ Units μg^{-1}) in Penaeus japonicus larvae reared using the following feeding regimes;

1. Live feeds; C. gracilis (50 - 125 cell μl^{-1}) throughout the larval stages, with the addition of 2-7 Artemia nauplii ml^{-1} during the mysis stages.

2. Artificial diet; PZ_1 to PZ_3 Frippak CAR (8mg l^{-1} day^{-1}), PZ_3 to M_2 Frippak CD2 (12mg l^{-1} day^{-1}), M_2 to PL_1 Frippak CD3 (16mg l^{-1} day^{-1}).

3. Mixed diet; Frippak diet regime as above, plus 10-50 cells μl^{-1} Chaetoceros gracilis through all larval stages.

N= 3-6 for each value, data in Appendix 5. Different superscripts denote significantly different values at each stage ($p<0.05$, Tukey's or Scheffe's tests for multiple comparisons or t-test for two sample comparisons).

Figure 3.

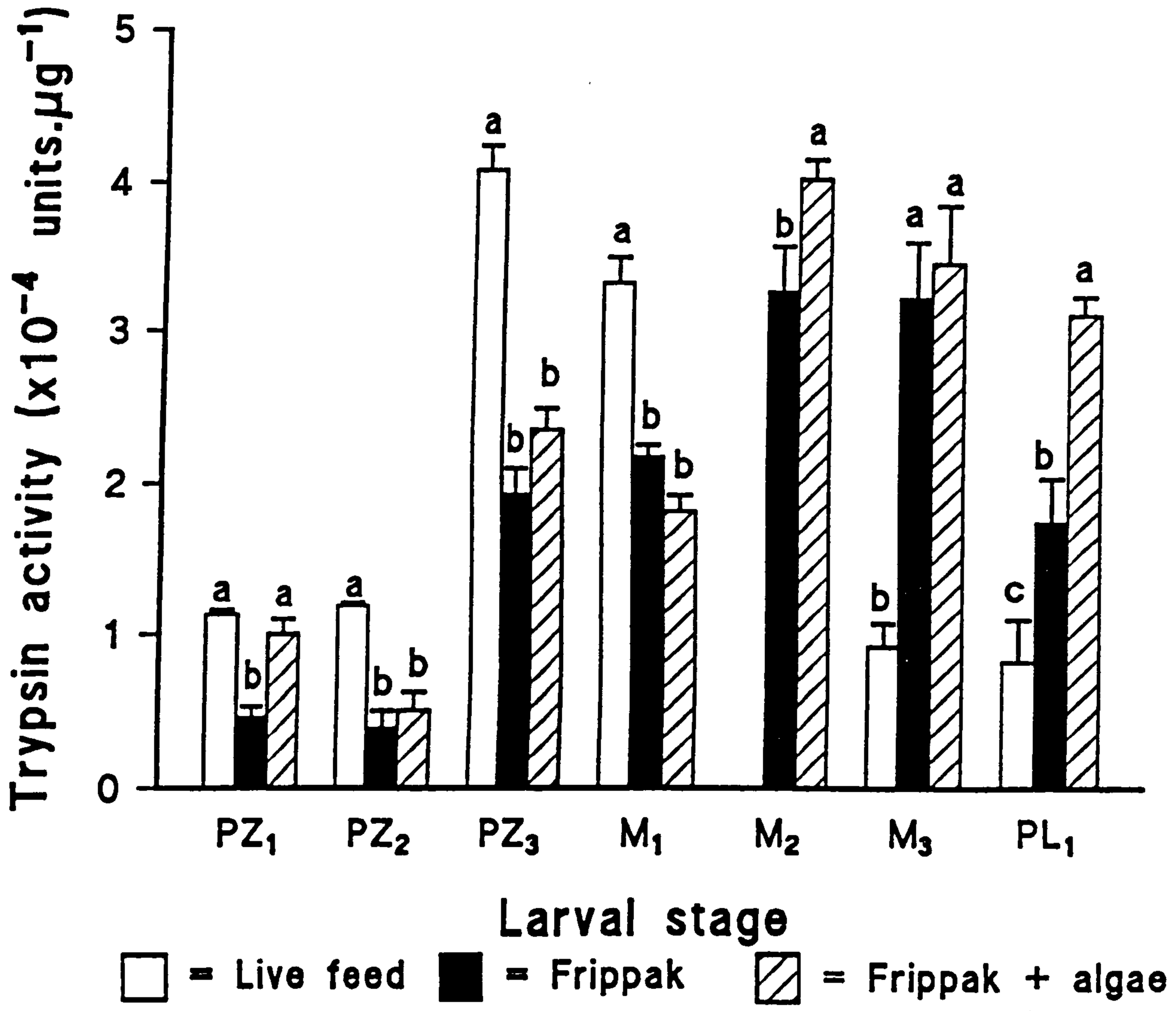

Trypsin activity (x10⁻⁴ units.µg⁻¹)
0
1
2
3
4
5
PZ1
PZ2
PZ3
M1
M2
M3
PL1
Larval stage
= Live feed
= Frippak
= Frippak + algae

Figure 4. Tissue amylase activity ($x10^{-5}$ Units μg^{-1}) in Penaeus japonicus larvae reared using the following feeding regimes;

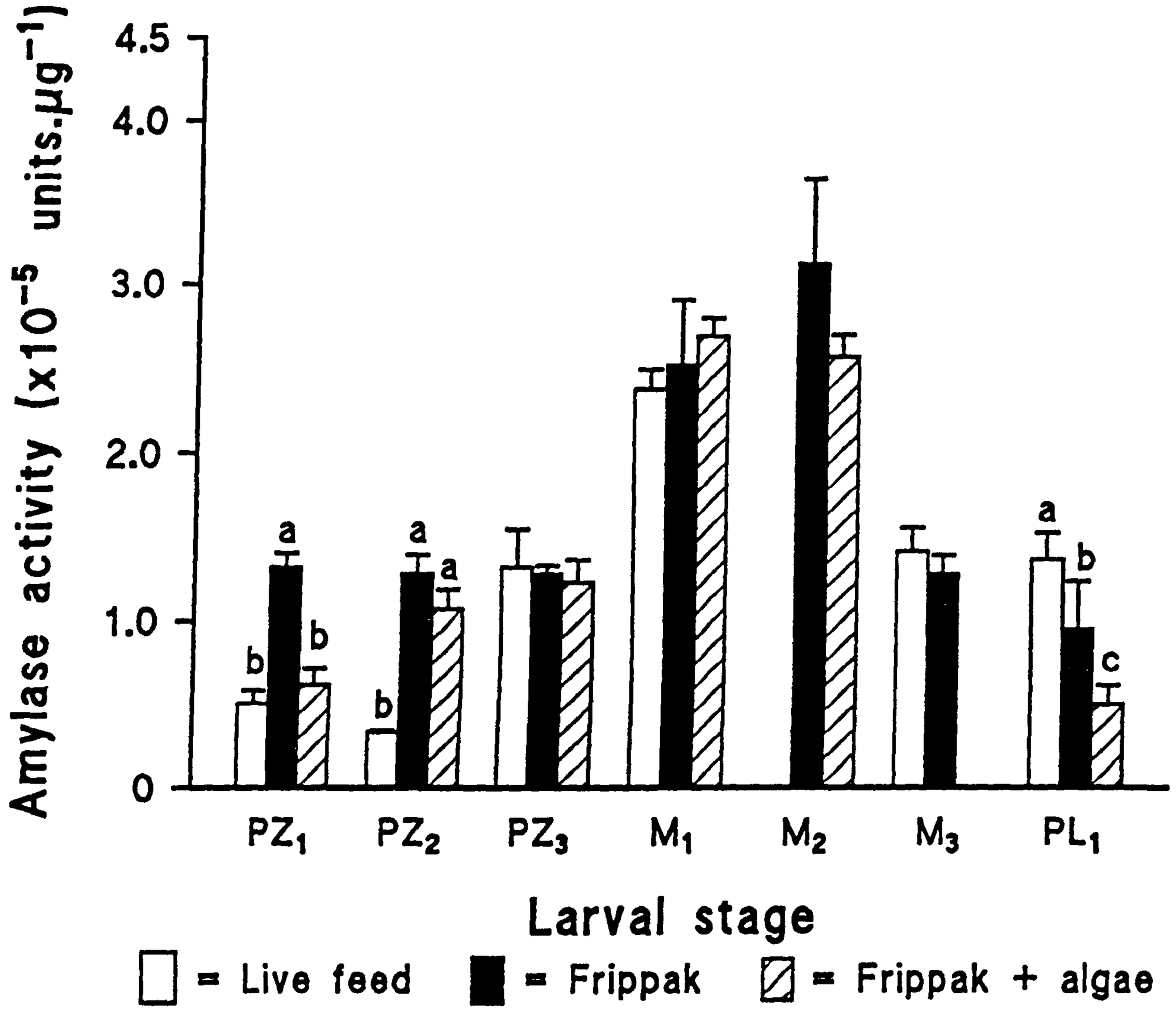
1. Live feeds; C. gracilis (50 - 125 cell μl^{-1}) throughout the larval stages, with the addition of 2-7 Artemia nauplii ml^{-1} during the mysis stages.

2. Artificial diet; PZ_1 to PZ_3 Frippak CAR (8mg l^{-1} day^{-1}), PZ_3 to M_2 Frippak CD2 (12mg l^{-1} day^{-1}), M_2 to PL_1 Frippak CD3 (16mg l^{-1} day^{-1}).

3. Mixed diet; Frippak diet regime as above, plus 10-50 cells μl^{-1} C. gracilis through all larval stages.

N= 3-6 for each value, data in Appendix 5. Different superscripts denote significantly different values at each stage.($p<0.05$, Tukey's or Scheffe's tests for multiple comparisons or t-test for two sample comparisons).

Figure 4.


Amylase activity (x10⁻⁵ units.µg⁻¹)
4.5
4.0
3.0
2.0
1.0
0
a
b
PZ1
PZ2
PZ3
M1
M2
M3
PL1
Larval stage
= Live feed
= Frippak
= Frippak + algae

Chapter 6

GENERAL CONCLUSIONS

In Chapter 2, it has been demonstrated that raising the lipid content of a spray-dried microencapsulated diet formulation to 33% lipid can significantly improve the accumulation of n-3 HUFA and total lipid in rotifers during secondary enrichment. Within the range of enrichment times and feeding levels tested no significant benefit, in terms of n-3 HUFA composition and total lipid content, was gained by feeding more than 0.5g of diet per million rotifers or by enrichment beyond 4h. Rotifer population decline during enrichment with the high lipid diet was similar to that previously recorded for similar diets of lower lipid content (20%), and indicates that a short enrichment period is optimal. Results with carp larvae indicate the effective transfer of at least some of the accumulated lipid in rotifers enriched with the high-lipid diet to fish larvae fed them. The improved length of carp larvae fed these rotifers is attributable to the increased percentage levels of n-3 HUFA, while a component of the observed improvement in weight may result from an energetic gain from extra lipid in the rotifers.

In Chapter 3 it has been shown that spray-dried diets, in common with other forms of microencapsulated particle, are unsuitable as feeds for first-feeding fish larvae due to their relatively poor digestibility. The feeding strategy of early fish larvae when presented with high prey densities, such as those used in aquaculture, is to process a large relatively amount of prey with a low assimilation efficiency. However, enough of the pool of soluble nutrients contained in live prey can be readily extracted so that increased ingestion results in maximisation of total energy assimilation. The poor digestive enzyme response and low ingestion rates exhibited by early carp larvae means that insufficient energy is extracted from artificial diets to support survival and growth equivalent to that achieved with live food. These results demonstrate that carp larvae are similar to stomachless marine fish larvae in the way that they process live and artificial foods, and that they can provide a useful model in the testing of the behaviour of diet particles in the larval fish gut.

The primary conclusion to be drawn from Chapters 4 and 5 is that the amount of dietary protein required to support growth in penaeid larvae is substantially lower than suggested by the composition of effective live and artificial food reviewed in the literature. In Chapter 5 the successful culture of Penaeus japonicus larvae through the protozoeal stages using Chaetoceros gracilis has shown that good growth may be achieved using an alga with protein content as low as 7% of dry weight. Furthermore, in Chapter 4, the same alga has been shown to support growth, albeit relatively poor, through the mysis stages to metamorphosis. Larvae feeding on C. gracilis exhibit high levels of trypsin activity, which may be in part a response to the low protein content of the alga as well as to direct stimulation of enzyme synthesis by an algal component. When a combination of Artemia nauplii and C. gracilis was used to feed

mysis larvae growth and protein retention were superior to when Artemia was used alone. This indicates a role for the algal diet in protein assimilation by mysis larvae fed zooplankton prey, which may well be mediated by improved trypsin secretion.

The performance of artificial diets for penaeid larvae can match that of live foods, particularly when used together with a low density of algal cells. In the early protozoeal stages growth on artificial diets may well be limited by low digestive enzyme responses and an algal co-feed might act to improve growth through stimulation of trypsin secretion. The observation of high trypsin activity, poor growth and low nitrogen content in mysis larvae fed a protein-rich artificial diet implies that only a small proportion of dietary protein content is digestible to the larvae. In the mysis stages, however, the improvement in trypsin activity in response to an algal co-feed with the artificial diet is small. In both protozoeal and mysis larvae, the alga may act by providing a source of more readily assimilated nutrients or might supply some factor, as yet unidentified, which improves the ability of larvae to meet their nutritional requirements for optimal growth.

The present study provides an opportunity to compare the feeding and digestive strategies of fish and penaeid shrimp larvae. At first-feeding penaeid larvae are better able to adapt to artificial diets than are fish larvae, despite the fact that neither type of larva is able to respond to an artificial diet with the increase in trypsin activity observed when live foods are ingested. Although the relative contribution of trypsin to total proteolytic activity is not known, data for tissue trypsin activity suggests that protease activity in PZ_1 P. japonicus fed the Frippak diet is only slightly higher than that in carp larvae fed a similar diet formulation (4.6×10^{-5} Units μg^{-1} compared to 1.44×10^{-5} Units μg^{-1}). Thus the more successful adaptation to artificial diets observed in PZ_1 penaeid larvae may result from slightly higher digestive enzyme activity, coupled with maintenance of, or increase in, ingestion rates compared to that of algal foods. Furthermore, mastication of the small size of particle ingested combined with mixing of food within the gut is likely to provide a greater surface area for enzymatic action. In contrast, the simple linear movement of intact larger diet particles through the fish larval gut limits digestive action to a relatively small surface area. The low ingestion rates typical of larval fish fed artificial diet, coupled with a low assimilation efficiency, result in low nutrient assimilation which may not be sufficient to meet maintenance requirements.

Comparison of trypsin activities in M_2 P. japonicus larvae and first-feeding carp larvae fed Artemia suggests that protease activity is similar (5.6×10^{-5} Units μg^{-1} compared to 4.31×10^{-5} Units μg^{-1}). At this stage ingestion of Artemia by penaeid larvae is relatively low (120% body weight day^{-1}), compared to the high rate of ingestion by first-feeding carp larvae (800% body weight day^{-1}). However, the

specific growth rate of mysis stage penaeid larvae is lower (10-15% day^{-1}, Kurmaly et al. 1989a) than that of early carp larvae (30-50% day^{-1}, Chapter 3) and normal growth can be supported at the lower ingestion rate. Mysis larvae can respond to artificial diets with much higher trypsin activities, allowing growth to be maintained even with a relatively low ingestion rate of a poorly digestible protein source. A similar response also develops in carp larvae after the first week of feeding, at which point effective utilisation of artificial diets becomes possible.

From the results of the present work, several key areas for the improvement of artificial diets for fish and penaeid larvae can be identified. In Chapter 3 it is suggested that low uptake of relatively indigestible protein limits growth in fish larvae fed artificial diets. The apparent protein-sparing effect observed in carp larvae fed rotifers of high lipid content suggests that there is scope for future research into lipid uptake from artificial diets, possibly examining inclusion levels, methods of preparation (emulsification) and use of lipase supplements. The inclusion of pancreatin (which contains lipases) into cross-linked protein microcapsules has been demonstrated to improve the digestibility of diet particles within the gut of first-feeding carp, and may offer a means to replace live prey in the delivery of soluble, readily digested nutrients to fish larvae. Alternatively, microcapsules containing a high level of pancreatin might be used as a binary feed to improve digestion of a standard microencapsulated diet. Whatever benefits are gained from improved digestibility, feeding regimes and culture systems must also be modified to maximise ingestion of artificial diets.

The spray-dried form of microcapsule is effective in providing a total replacement for live food for penaeid larvae. However current artificial diets are far from ideal in the effective delivery of readily digestible protein and may lack digestive stimulants and other nutritional factors. The identification of algal components which stimulate growth in protozoea might yield benefits in diet development. Another approach would be to test a range of algal ingredients which might stimulate digestive enzyme production and reduce the need for an algal co-feed. Although cultured phytoplankton tends to be prohibitively expensive, dried products such as Tetraselmis sp. and Dunaliella salina meal are commercially available. Cheaper alternatives such as Spirulina might also be used and have been found to stimulate trypsin activity in Penaeus indicus PZ_1 larvae (L. Le Vay and D.A. Jones, unpublished data).

Research into improvement of artificial diets for mysis stage penaeid larvae will need to focus on the balancing of low diet leaching properties with higher digestibility. This presents problems similar to, but not as extreme as, those encountered with fish larvae. Although inclusion of digestive enzymes into penaeid

diets might be yield benefits, such an approach is unlikely to be feasible. Penaeid larvae are encounter feeders and a high density of diet particles must be maintained in suspension if effective feeding is to take place. Thus diets rendered unstable by enzyme inclusion will inevitable have a deleterious effect on water quality. A more promising approach would be to examine the effect of different inclusion levels of more digestible protein sources on leaching rates, trypsin activity and growth.

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APPENDIX 1: Sources of materials used.

Sigma Chemical Company, Poole, Dorset.

BAPNA (Nα-benzoyl-L-argenine-p-nitroanilide)
BHT (butylated hydroxytoluene)
Boron triflouride
Calcium chloride
Dimethyl sulphoxide
3,5,- dinitrosalicylic acid
Haemoglobin (bovine)
MS-222
Nonadecanoic acid (>99% pure)
Pancreatin (porcine 8x U.S.P)
Potassium bicarbonate
Potassium chloride
Succinyl dichloride
Sodium chloride
Sodium hydroxide
Sodium phosphate
Sodium potassium tartrate
Sodium silicate
Sulphuric acid (analar grade)
TAME (Nα-p-toluenesulphonyl-L-argenine methyl ester)
Total protein test kit (Lowry method)
Trizma base
Trichloroacetic acid
Tricosanoic acid
Tyrosine

Aldrich Chemical Company, Gillingham, Dorset

Cyclohexanone 2,4-dinitrophenylhydrozone

Merck/BDH

Cyclohexane
Maltose monohydrate
Soluble starch

Boehringer Manheim, Uckfield, E. Sussex.

Total lipid test kit

APPENDIX 2: Statistical tables and data for figures and tables in Chapter 2.

Two-way ANOVA for 20.5n-3 contents of rotifers from three dietary treatments and four sampling times (data taken from Table 1, Chap. 2).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Diet	2	62.439	62.439	31.219	21.63	0.000
time	3	5.335	0.867	0.289	0.20	0.895
diet*time	6	13.727	13.727	2.288	1.59	0.215
Error	16	23.091	23.091	1.443		
Total	27	104.591				

Two-way ANOVA for 22.6n-3 contents of rotifers from three dietary treatments and four sampling times (data taken from Table 1, Chap. 2).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
diet	2	123.605	123.605	61.803	38.03	0.000
time	3	5.412	3.579	1.193	0.73	0.547
diet*time	6	13.023	13.023	2.171	1.34	0.298
Error	16	26.002	26.002	1.625		
Total	27	168.043				

Two-way ANOVA for total n-3 HUFA contents of rotifers from three dietary treatments and four sampling times (data taken from Table 1, Chap. 2).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
diet	2	349.938	349.938	174.969	21.17	0.000
time	3	31.795	11.603	3.868	0.47	0.709
diet*time	6	61.474	61.474	10.246	1.24	0.338
Error	16	132.238	132.238	8.265		
Total	27	575.445				

Two-way ANOVA for 20.5n-3 contents of rotifers from two dietary treatments fed at three densities. (data taken from Table 1, Chap. 2).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
diet	1	9.754	9.754	9.754	5.77	0.027
level	2	4.439	4.439	2.219	1.31	0.294
diet*level	2	0.347	0.347	0.174	0.10	0.903
Error	18	30.435	30.435	1.691		
Total	23	44.974				

Two-way ANOVA for 22.6n-3 contents of rotifers from two dietary treatments fed at three densities. (data taken from Table 1, Chap. 2).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
diet	1	85.051	85.051	85.051	45.71	0.000
level	2	3.055	3.055	1.528	0.82	0.456
diet*level	2	1.521	1.521	0.760	0.41	0.671
Error	18	33.490	33.490	1.861		
Total	23	123.117				

APPENDIX 2(continued)

Two-way ANOVA for total n-3 contents of rotifers from two dietary treatments (CAR and Booster) fed at three densities. (data taken from Table 1, Chap. 2).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
diet	1	163.856	163.856	163.856	16.62	0.001
level	2	13.945	13.945	6.972	0.71	0.506
diet*level	2	5.192	5.192	2.596	0.26	0.771
Error	18	177.472	177.472	9.860		

Table 3.

One way ANOVA for total lipid data (% dry weight) for 9 day old carp larvae pooled from three replicates of each of three dietary treatments.

SOURCE	DF	SS	MS	F	p
FACTOR	2	4.258	2.129	2.47	0.118
ERROR	15	12.921	0.861		
TOTAL	17	17.179			

Figure 1a.

One way ANOVA of 20.5 n-3 content of rotifers from three dietary treatments, data pooled over feeding level and duration (from Table 1, Chap. 2).

SOURCE	DF	SS	MS	F	p
diet	2	62.44	31.22	18.52	0.000
ERROR	25	42.15	1.69		
TOTAL	27	104.59			

One way ANOVA of 22.6n-3 content of rotifers from three dietary treatments, data pooled over feeding level and duration (from Table 1, Chap. 2).

SOURCE	DF	SS	MS	F	p
diet	2	123.61	61.80	34.77	0.000
ERROR	25	44.44	1.78		
TOTAL	27	168.04			

One way ANOVA of total n-3 HUFA content of rotifers from three dietary treatments, data pooled over feeding level and duration (from Table 1, Chap. 2).

SOURCE	DF	SS	MS	F	p
diet	2	349.94	174.97	19.40	0.000
ERROR	25	225.51	9.02		
TOTAL	27	575.44			

APPENDIX 2(continued)

Figure 1b.

Total lipid (% of dry weight) data for rotifers fed Frippak Booster over a 16h period.

0h	4h	8h	16h
13.06	24.13	18.67	19.63
12.70	18.59	18.16	18.57
11.55	23.35	19.37	19.12
13.06	20.36	18.54	18.23
13.98	20.33	18.45	18.29
11.48	18.46	19.55	17.21
13.17	20.17	18.70	18.44
12.73	19.68	17.68	18.95
12.00	21.57	18.87	19.34
13.59	20.65	18.40	18.25
12.37	23.52	18.92	18.79
12.54	19.88	21.61	17.64

Figure 2.

Analysis of covariance for rotifer numbers in three replicates with time for treatment fed Frippak Booster.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
REPS	2	137.09	157.61	78.80	2.64	0.077
TIME	1	563.55	563.55	563.55	18.90	0.000
REPS*TIME	2	141.72	141.72	70.86	2.38	0.099
Error	84	2504.80	2504.80	29.82		
Total	89	3347.16				

Analysis of covariance for rotifer numbers in three replicates with time for unfed control treatment.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
REPS	2	29.60	15.09	7.55	0.30	0.741
TIME	1	9.58	9.58	9.58	0.38	0.538
REPS*TIME	2	26.52	26.52	13.26	0.53	0.592
Error	84	2108.70	2108.70	25.10		
Total	89	2174.40				

Regression of rotifer counts (in 50μl) vs. time (h) for enriched treatment (data pooled from three replicates)

Predictor	Coef	Stdev	t-ratio	p
Intercept	27.980	1.063	26.31	0.000
Time	-0.5065	0.1058	-4.79	0.000

s = 5.324 R-sq = 23.9% R-sq(adj) = 22.8%

SOURCE	DF	SS	MS	F	p
Regression	1	649.51	649.51	22.91	0.000
Error	73	2069.24	28.35		
Total	74	2718.75			

Figure 2 (continued).

Regression of rotifer counts (in 50μl) vs. time (h) for unfed control treatment (data pooled from three replicates).

Predictor	Coef	Stdev	t-ratio	p
Constant	28.3481	0.9488	29.88	0.000
Time	0.04210	0.09441	0.45	0.657

s = 4.751 R-sq = 0.3% R-sq(adj) = 0.0%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	4.49	4.49	0.20	0.657
Error	73	1647.46	22.57		
Total	74	1651.95			

Figure 3.

Two way ANOVA for length data (mm) for 9 day old carp larvae from three replicates of each of three dietary treatments.

Source	DF	Seq SS	Adj SS	Adj MS	F	p
diets	2	10.6680	10.6680	5.3340	16.62	0.000
reps	2	0.6598	0.6598	0.3299	1.03	0.362
diets*reps	4	0.3078	0.3078	0.0769	0.24	0.915
Error	81	25.9935	25.9935	0.3209		
Total	89	37.6291				

One way ANOVA for length data (mm) for 9 day old carp larvae pooled from three replicates of each of three dietary treatments.

SOURCE	DF	SS	MS	F	p
diets	2	10.668	5.334	17.21	0.000
ERROR	87	26.961	0.310		
TOTAL	89	37.629			

Nested ANOVA for weight data (mg) for 9 day old carp larvae from three replicates of each of three dietary treatments.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
treat	2	1104.29	1104.29	552.14	29.48	0.000
rep	2	5.69	5.69	2.84	0.15	0.859
treat*rep	4	9.51	9.51	2.38	0.13	0.972
Error	81	1517.00	1517.00	18.73		
Total	89	2636.49				

APPENDIX 3: Statistical tables and data for figures and tables in Chapter 3.

Figure 2a.

Analysis of covariance for length (mm) of postlarval carp fed Artemia or artificial diet

Source	DF	Seq SS	Adj SS	Adj MS	F	P
DAYS	1	4173.16	4173.16	4173.16	2531.48	0.000
DIET	1	1762.20	35.04	35.04	21.26	0.000
REPS(DIET)	4	79.58	4.18	1.04	0.63	0.639
DIET*DAYS	1	693.44	693.44	693.44	420.65	0.000
REPS*DAYS(DIET)	4	9.58	9.58	2.40	1.45	0.216
Error	438	722.05	722.05	1.65		
Total	449	7440.02				

Term			Coeff	Stdev	t-value	P
Constant			14.3056	0.1419	100.78	0.000
DAYS			1.07667	0.02140	50.31	0.000
DAYS*DIET						
	1		0.43889	0.02140	20.51	0.000
DAYS*(DIET)REPS						
	1	1	-0.01889	0.04280	-0.44	0.659
	1	2	0.04778	0.04280	1.12	0.265
	2	1	0.06556	0.04280	1.53	0.126
	2	2	-0.08778	0.04280	-2.05	0.041

Figure 2b.

Analysis of covariance for ln weight (mg) of postlarval carp fed Artemia or artificial diet

Source	DF	Seq SS	Adj SS	Adj MS	F	P
days	1	11.4937	11.4937	11.4937	1895.72	0.000
treat	1	1.5978	0.0009	0.0009	0.15	0.706
rep(treat)	4	0.2908	0.0428	0.0107	1.77	0.180
treat*days	1	0.7349	0.7349	0.7349	121.21	0.000
rep*days(treat)	4	0.0273	0.0273	0.0068	1.13	0.375
Error	18	0.1091	0.1091	0.0061		
Total	29	14.2537				

Term			Coeff	Stdev	t-value	P
Constant			3.80328	0.02462	154.46	0.000
days			0.218838	0.005026	43.54	0.000
days*treat						
	1		0.055337	0.005026	11.01	0.000
days*(treat)rep						
	1	1	-0.00620	0.01005	-0.62	0.545
	1	2	0.00853	0.01005	0.85	0.407
	2	1	0.01175	0.01005	1.17	0.258
	2	2	-0.01929	0.01005	-1.92	0.071

APPENDIX 3 (continued)

Figure 3b.
Length (mm) of carp larvae reared using Artemia nauplii or Frippak CD artificial diet (first experiment)

Initial (Day 0)			Frippak CD Day 6		
0.71	0.72	0.73	7.82	7.97	8.44
0.72	0.69	0.73	8.13	7.66	8.44
0.74	0.66	0.69	8.13	7.97	8.28
0.70	0.71	0.74	8.28	7.66	8.13
0.69	0.71	0.70	8.28	8.28	8.44
0.68	0.70	0.69	8.44	8.28	7.97
0.68	0.72	0.71	8.13	9.22	8.28
0.71	0.70	0.73	8.60	8.13	8.13
0.68	0.72	0.70	8.13	8.60	7.82
0.72	0.64	0.71	7.82	7.82	8.28

Artemia Day 6			Artemia Day 11		
10.02	9.62	9.02	18.79	18.79	18.79
9.39	9.74	9.74	18.79	17.54	17.54
9.55	9.82	9.52	18.79	17.54	15.03
9.38	9.65	9.35	20.04	16.29	16.29
9.42	9.45	9.45	16.29	17.54	18.79
9.35	9.38	9.38	16.29	17.54	20.04
9.40	9.03	8.81	18.79	17.54	18.79
9.15	9.30	9.30	17.54	18.79	17.54
9.67	9.43	9:33	17.54	18.79	16.29
9.08	8.92	9.42	18.79	17.54	18.79

Frippak CD Day 11

9.22, 10.15, 8.59, 8.59, 9.37, 8.43, 9.22, 8.59, 8.59

Final weights (mg) of carp larvae reared for 11 days on Artemia (1st expt).

41.9, 47.9, 34.2, 60.8, 35.4, 33, 49.4, 38.4, 27.4, 40.9, 38.9, 48.4, 38.2, 47.3, 37.1, 32.4, 29.1, 34.9, 37.2, 47.3

Final weights (mg) of carp larvae reared for 11 days on Frippak (1st expt)

2 2 2 2 3 3 3 3 4

Figure 4.

Final weights (mg) of carp larvae reared for 10 days on Frippak (2nd expt).

9 5 2 6 7 10 5 3 4 4 5 8 5 9 3

Final lengths (mm) of carp larvae reared for 10 days on Frippak (2nd expt).

11.08 8.74 7.64 10.45 10.30 10.45 9.20 10.14 9.36 10.45 10.14 7.96 8.42 7.96 9.98

APPENDIX 3 (continued)

Figure 6.

Linear regression of position of food boundary against time 2mg larvae continuously feeding on Artemia nauplii

Position = 98.1 - 1.04 time(min)

Predictor	Coef	Stdev	t-ratio	p
Constant	98.137	4.278	22.94	0.000
C2	-1.03810	0.08472	-12.25	0.000

s = 12.28 R-sq = 79.8% R-sq(adj) = 79.3%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	22631	22631	150.14	0.000
Error	38	5728	151		
Total	39	28358			

Linear regression of position of food boundary against time 2mg larvae continuously feeding on artifical diet nauplii

Position = 93.5 - 1.00 time (min)

Predictor	Coef	Stdev	t-ratio	p
Constant	93.493	5.544	16.86	0.000
C2	-1.0019	0.1098	-9.13	0.000

s = 15.91 R-sq = 68.7% R-sq(adj) = 67.8%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	21081	21081	83.29	0.000
Error	38	9618	253		
Total	39	30699			

Analysis of covariance for position of food boundary for 2mg larvae continuous feeding on Artemia nauplii or artifical diet.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Time	1	43698	43698	43698	216.42	0.000
Diet	1	182	89	89	0.44	0.509
Time*diet	1	14	14	14	0.07	0.795
Error	76	15346	15346	202		
Total	79	59239				

APPENDIX 3 (continued)

Figure 7a.

Trypsin activity (x 10^{-3} units $larva^{-1}$) in carp larvae kept under three dietary regimes for 24h at different stages of development.

	Artemia	CD	Unfed
1-2mg	8.63 ± 1.51	2.87 ± 1.02	3.16 ± 0.72
4-5mg	6.35 ± 1.20	11.05 ± 0.87	2.70 ± 0.47
5-6mg	14.23 ± 1.00	29.06 ± 3.44	7.64 ± 1.71
9-10mg	11.05 ± 0.69	10.69 ± 2.37	9.67 ± 1.40
12-14mg	72.85 ± 11.94	59.51 ± 6.14	21.52 ± 4.60

Figure 7b.

Trypsin activity (units/individual) in carp postlarvae fed Artemia or artificial diet.

Artemia		Artificial	
Weight (mg)	Activity	Weight (mg)	Activity
14	0.072 ± 0.011	13	0.059 ± 0.006
35	0.231 ± 0.018	34	0.592 ± 0.055
55	0.659 ± 0.089	51	0.936 ± 0.078
76	0.477 ± 0.029	60	0.913 ± 0.019
89	0.910 ± 0.046	77	1.159 ± 0.039
100	0.890 ± 0.032	84	1.394 ± 0.046

Analysis of covariance for trypsin activity in postlavae fed Artemia or artifical diet.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Weight	1	1.21836	1.54638	1.54638	93.81	0.000
Diet	1	0.48667	0.00025	0.00025	0.02	0.905
Diet*Weight	1	0.12401	0.12401	0.12401	7.52	0.025
Error	8	0.13187	0.13187	0.01648		
Total	11	1.96091				

Term	Coeff	Stdev	t-value	P
Constant	-0.06199	0.08669	-0.72	0.495
Weight	0.013410	0.001384	9.69	0.000
Weight*Diet				
1	-0.003797	0.001384	-2.74	0.025

APPENDIX 3 (continued)

Figure 8a.

Total protease activity (units $larva^{-1}$) in carp larvae kept under three dietary regimes for 24h at different stages of development.

	Artemia	CD
1-2mg	0.143 ± 0.014	0.092 ± 0.006
4-5mg	0.602 ± 0.042	1.102 ± 0.110
5-6mg	1.195 ± 0.085	1.879 ± 0.138
9-10mg	0.096 ± 0.091	1.341 ± 0.128
12-14mg	1.947 ± 0.011	1.758 ± 0.169

Figure 9a.

Trypsin activity in 2 mg carp larvae during change of diet from Artemia to Frippak CD and vice-versa.

Time (h)	Activity (x 10^{-3} Units $larva^{-1}$)	
	CD - Artemia	Artemia - CD
0	1.16 ± 0.21	3.52 ± 0.21
2	2.08 ± 0.48	3.06 ± 0.41
4	2.42 ± 0.16	1.23 ± 0.46
10	3.30 ± 0.24	1.83 ± 0.45
Unfed	0.52 ± 0.64	

Figure 9b.

Trypsin activity in 4 mg carp larvae during weaning from Artemia to Frippak CD.

Time (h)	Activity (x 10^{-3} Units $larva^{-1}$)
0	3.80 ± 1.78
2	3.96 ± 1.23
5	6.63 ± 1.62
10	9.17 ± 2.05
Unfed	3.32 ± 0.21

APPENDIX 3 (continued)

Figure 8a.

Total protease activity (units $larva^{-1}$) in carp larvae kept under three dietary regimes for 24h at different stages of development.

	Artemia	CD
1-2mg	0.143 ± 0.014	0.092 ± 0.006
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5-6mg	1.195 ± 0.085	1.879 ± 0.138
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Figure 9a.

Trypsin activity in 2 mg carp larvae during change of diet from Artemia to Frippak CD and vice-versa.

Time (h)	Activity (x 10^{-3} Units $larva^{-1}$)	
	CD - Artemia	Artemia - CD
0	1.16 ± 0.21	3.52 ± 0.21
2	2.08 ± 0.48	3.06 ± 0.41
4	2.42 ± 0.16	1.23 ± 0.46
10	3.30 ± 0.24	1.83 ± 0.45
Unfed	0.52 ± 0.64	

Figure 9b.

Trypsin activity in 4 mg carp larvae during weaning from Artemia to Frippak CD.

Time (h)	Activity (x 10^{-3} Units $larva^{-1}$)
0	3.80 ± 1.78
2	3.96 ± 1.23
5	6.63 ± 1.62
10	9.17 ± 2.05
Unfed	3.32 ± 0.21

APPENDIX 3 (continued)

Table 1a.
Dry weights (mg) of carp larvae of approximately 2 mg wet weight before and after 3h feeding on Frippak CD3 or Artemia nauplii

Prefeed	CD	Artemia
0.320	0.333	0.360
0.170	0.367	0.301
0.289	0.330	0.384
0.302	0.336	0.370
0.238	0.300	0.465
0.321	0.356	0.434
0.232	0.302	0.244
0.302	0.338	0.395
0.260	0.383	0.301
0.206	0.280	0.456
0.240	0.208	0.381
0.312	0.422	0.380
0.331	0.376	0.267
0.257	0.352	0.412
0.232	0.257	0.380
0.248	0.496	0.332
0.274	0.326	0.311
0.272	0.357	0.458
0.233		0.368
0.225		0.484

TWOSAMPLE T FOR CD VS prefeed

	N	MEAN	STDEV	SE MEAN
caps	18	0.3399	0.0628	0.015
prefeed	20	0.2632	0.0430	0.0096

95 PCT CI FOR MU caps - MU prefeed: (0.041, 0.1128)

TTEST MU caps = MU prefeed (VS NE): T= 4.35 P=0.0002 DF= 29

TWOSAMPLE T FOR artemia VS prefeed

	N	MEAN	STDEV	SE MEAN
artemia	20	0.3741	0.0668	0.015
prefeed	20	0.2632	0.0430	0.0096

95 PCT CI FOR MU artemia - MU prefeed: (0.075, 0.1471)

TTEST MU artemia = MU prefeed (VS NE): T= 6.24 P=0.0000 DF=32

APPENDIX 3 (continued)

Table 1b.

Dry weights (mg) of carp larvae of approximately 4 mg wet weight before and after 3h feeding on Frippak CD3 or Artemia nauplii

Prefeed	CD	Artemia
0.584	0.738	0.832
0.681	0.841	0.705
0.467	0.637	0.679
0.587	0.661	0.851
0.531	0.601	0.858
0.624	0.711	0.584
0.586	0.779	0.726
0.444	0.668	0.630
0.448	0.646	0.971
0.401	0.826	0.574
0.406	0.838	0.685
0.489	0.824	0.975
0.544	0.742	0.548
0.429	0.517	0.695
0.620	0.859	0.576
0.526	0.871	0.647
0.488	0.994	0.722
0.613	0.527	0.568
0.402	0.715	0.800

TWOSAMPLE T FOR CAP 4 VS UNFED 4

	N	MEAN	STDEV	SE MEAN
CD	20	0.739	0.121	0.027
UNFED	20	0.5230	0.0853	0.019

95 PCT CI FOR MU CAP 4 - MU UNFED 4: (0.149, 0.283)

TTEST MU CAP 4 = MU UNFED 4 (VS NE): T= 6.51 P=0.0000 DF= 34

TWOSAMPLE T FOR ART 4 VS UNFED 4

	N	MEAN	STDEV	SE MEAN
ART	20	0.715	0.129	0.029
UNFED	20	0.5230	0.0853	0.019

95 PCT CI FOR MU ART 4 - MU UNFED 4: (0.121, 0.262)

TTEST MU ART 4 = MU UNFED 4 (VS NE): T= 5.54 P=0.0000 DF= 32

APPENDIX 4: Data from figures presented in Chapter 4.

Figure 1.

Total trypsin activity (units larva^{-1}) in larvae from three dietary treatments.

	C. gracilis	Artemia	mixed
M_1	0.01682 ± 0.00114	0.00856 ± 0.0016	0.00687 ± 0.0007
M_2	0.01445 ± 0.00081	0.00226 ± 0.0005	0.0073 ± 0.0007
M_3	0.01645 ± 0.00185	0.00321 ± 0.0002	0.01407 ± 0.0017
PL_1	0.011 ± 0.00131	0.00509 ± 0.0007	0.00438 ± 0.0009

Figure 2.

Total amylase activity (x x 10^{-5} units larva^{-1}) in larvae from three dietary treatments.

	C. gracilis	Artemia	mixed
M_1	88.86 ± 2.89	38.52 ± 10.75	45.09 ± 2.444
M_2	114.42 ± 4.34	41.18 ± 6.34	70.83 ± 17.38
M_3	40.33 ± 1.548	43.05 ± 2.313	54.58 ± 0.481
PL_1	81.20 ± 40.	80.15 ± 10.61	58.08 ± 5.72

APPENDIX 5: Statistical tables and data for figures and tables presented in Chapter 5

Table 4.

Length data; analysis of covariance, day 1 to day 5

Source	DF	Seq SS	Adj SS	Adj MS	F	P
tube	6	0.6138	0.3585	0.0598	9.17	0.000
day	1	57.1342	51.1937	51.1937	7853.84	0.000
tube*day	6	0.2921	0.2921	0.0487	7.47	0.000
Error	470	3.0636	3.0636	0.0065		
Total	483	61.1037				

Term	Coeff	Stdev	t-value	P
Constant	6.71610	0.00902	744.96	0.000
day	0.241539	0.002726	88.62	0.000
day*tube				
Frippak1	0.017996	0.007481	2.41	0.017
Frippak2	0.015239	0.007348	2.07	0.039
Live 1	0.007092	0.007348	0.97	0.335
Live 2	-0.037351	0.007380	-5.06	0.000
Live 3	-0.018449	0.005541	-3.33	0.001
HWCAR 8	0.010748	0.005596	1.92	0.055

Table 5.

Length data; analysis of covariance day 5 to day 8

Source	DF	Seq SS	Adj SS	Adj MS	F	P
C41	6	0.57181	0.19869	0.03311	6.93	0.000
C42	1	2.59868	2.27664	2.27664	476.47	0.000
C41*C42	6	0.13710	0.13710	0.02285	4.78	0.000
Error	376	1.79660	1.79660	0.00478		
Total	389	5.10420				

Term	Coeff	Stdev	t-value	P
Constant	7.53453	0.02204	341.83	0.000
C42	0.072757	0.003333	21.83	0.000
C42*C41				
Frippak 1	-0.003714	0.009092	-0.41	0.683
Frippak 2	-0.005353	0.009280	-0.58	0.564
Live 1	-0.029078	0.008909	-3.26	0.001
Live 2	0.035079	0.008950	3.92	0.000
Live 3	0.008154	0.006726	1.21	0.226
HWCAR 1	0.008684	0.006800	1.28	0.202

APPENDIX 5 (continued).

Table 6.

Survival data; analysis of covariance day 1 to day 5

Source	DF	Seq SS	Adj SS	Adj MS	F	P
C31	6	15.2257	0.2502	0.0417	0.48	0.822
C32	1	2.1351	2.1351	2.1351	24.67	0.000
C31*C32	6	1.1220	1.1220	0.1870	2.16	0.047
Error	266	23.0237	23.0237	0.0866		
Total	279	41.5065				

Term		Coeff	Stdev	t-value	P
Constant		2.21975	0.05778	38.42	0.000
C32		-0.07810	0.01573	-4.97	0.000
C32*C31					
Frippak	1	0.02512	0.03852	0.65	0.515
Frippak	2	0.02268	0.03852	0.59	0.557
Live	1	-0.00976	0.03852	-0.25	0.800
Live	2	-0.07848	0.03852	-2.04	0.043
Live	3	-0.08118	0.03852	-2.11	0.036
HWCAR	1	0.03788	0.03852	0.98	0.326

Table 7.

Survival data; analysis of covariance from day 5 to day 8.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
C31	6	39.0180	0.7992	0.1332	1.21	0.300
C32	1	5.9136	5.9389	5.9389	54.05	0.000
C31*C32	6	3.5309	3.5309	0.5885	5.36	0.000
Error	264	29.0056	29.0056	0.1099		
Total	277	77.4681				

Term		Coeff	Stdev	t-value	P
Constant		2.6591	0.1172	22.69	0.000
C32		-0.13077	0.01779	-7.35	0.000
C32*C31					
Frippak	1	0.06474	0.04343	1.49	0.137
Frippak	2	0.07486	0.04343	1.72	0.086
Live	1	-0.04014	0.04441	-0.90	0.367
Live	2	-0.22431	0.04343	-5.17	0.000
Live	3	0.00743	0.04343	0.17	0.864
HWCAR	1	0.02686	0.04343	0.62	0.537

APPENDIX 5 (continued).

Figure 1.

Dry weight and length data for each larval stage from three dietary treatments.

Dry weight

(μg, mean ± s.d., n= 3 or 4)

Stage	Live food	Frippak	Frippak + alga
PZ_1	6.06 ± 0.05	4.83 ± 0.04	6.16 ± 0.20
PZ_2	19.31 ± 2.27	14.96 ± 1.87	18.16 ± 1.66
PZ_3	27.17 ± 2.85	27.91 ± 2.97	28.61 ± 1.60
M_1	38.72 ± 3.51	30.80 ± 2.54	37.18 ± 3.15
M_2	42.96 ± 0.93	35.96 ± 1.71	42.56 ± 1.96
M_3	49.50 ± 1.97	39.36 ± 0.80	46.43 ± 3.00
PL_1	73.67 ± 2.68	57.53 ± 1.59	72.54 ± 1.61

Length

(mm, mean ± s.d., n=20 for each value)

Stage	(A)	(B)	(C)
PZ_1	1.17 ± 0.05	1.04 ± 0.02	1.11 ± 0.05
PZ_2	1.89 ± 0.07	1.63 ± 0.06	1.85 ± 0.06
PZ_3	2.52 ± 0.09	2.54 ± 0.08	2.62 ± 0.08
M_1	3.34 ± 0.11	2.86 ± 0.11	3.34 ± 0.10
M_2	3.77 ± 0.10	3.05 ± 0.10	3.68 ± 0.10
M_3	3.98 ± 0.16	3.71 ± 0.15	3.82 ± 0.18
PL_1	4.65 ± 0.16	3.94 ± 0.10	4.53 ± 0.14

APPENDIX 5 (continued).

Figure 2.

Nitrogen and carbon data for each larval stage from three treatments.

Nitrogen

	Live food			Frippak			Frippak + alga		
	µg	s.d	(%)	µg	s.d.	(%)	µg	s.d.	(%)
PZ_1	31.3	± 1.4	(5.2)	36.0	± 0.9	(7.5)	36.1	± 3.5	(5.9)
PZ_2	111.7	± 9.0	(5.8)	96.2	± 7.8	(6.4)	115.4	± 19.4	(6.4)
PZ_3	195.4	± 24.7	(7.2)	194.5	± 10.3	(7.0)	199.8	± 22.6	(7.1)
M_1	325.0	± 9.2	(8.4)	235.2	± 2.0	(7.6)	319.4	± --	(8.6)
M_2	281.0	± 24.1	(6.5)	253.1	± 12.2	(7.0)	262.4	± 3.6	(6.2)
M_3	463.5	± 7.5	(9.4)	280.6	± 30.5	(7.1)	389.8	± 2.6	(8.1)
PL_1	653.5	± ---	(8.9)	371.5	± 28.3	(6.5)	642.9	± 25.1	(8.9)

Carbon

	Live food			Frippak			Frippak + alga		
	µg	s.d.	(%)	µg	s.d.	(%)	µg	s.d.	(%)
PZ_1	145	± 16	(23.9)	158	± 2	(32.7)	160	± 12	(26.0)
PZ_2	471	± 42	(24.4)	448	± 55	(30.0)	535	± 123	(29.4)
PZ_3	856	± 89	(31.5)	896	± 33	(32.1)	899	± 61	(24.4)
M_1	1305	± 23	(33.7)	1028	± 28	(33.4)	1350	± 123	(36.3)
M_2	1313	± 35	(30.6)	1134	± 41	(31.5)	1159	± 26	(27.2)
M_3	1824	± 22	(36.8)	1242	± 45	(31.5)	1711	± 21	(36.8)
PL_1	2683	± 64	(36.4)	1820	± 209	(31.6)	2642	± 63	(36.4)

APPENDIX 5 (continued).

Figure 3.

Trypsin activity ($x10^{-4}$ units μg^{-1}) in larvae from three dietary treatments.

	Live food	Frippak	Frippak + alga
PZ_1	1.184 ± 0.132	0.460 ± 0.068	1.014 ± 0.196
PZ_2	1.183 ± 0.025	0.388 ± 0.109	0.499 ± 0.116
PZ_3	4.082 ± 0.162	1.933 ± 0.170	2.357 ± 0.139
M_1	3.327 ± 0.171	2.067 ± 0.273	1.814 ± 0.113
M_1	n.d.	3.275 ± 0.295	4.027 ± 0.132
M_2	0.921 ± 0.153	3.234 ± 0.370	3.459 ± 0.393
M_3	0.822 ± 0.279	1.752 ± 0.293	2.974 ± 0.380
PL_1			

Amylase activity ($x10^{-5}$ units μg^{-1}) in larvae from three dietary treatments.

	Live food	Frippak	Frippak + alga
PZ_1	0.503 ± 0.079	1.323 ± 0.082	0.613 ± 0.098
PZ_2	0.331 ± 0.011	1.284 ± 0.111	1.066 ± 0.181
PZ_3	1.316 ± 0.227	1.280 ± 0.045	1.224 ± 0.138
M_1	2.373 ± 0.115	2.520 ± 0.383	2.686 ± 0.060
M_2	n.d.	3.133 ± 0.517	2.568 ± 0.157
M_3	1.421 ± 0.134	1.285 ± 0.109	n.d.
PL_1	1.371 ± 0.155	0.956 ± 0.280	0.500 ± 0.113

ACCOMPANYING MATERIAL

Jones, D.A., Le Vay, L. and Kamarudin, M.S. (1993). Feeding and nutritional requirements of penaeid shrimp larvae. In: Caldéron, J. and Sandoval, V. (editors), Mems. 1er congreso ecuatoriano de acuicultura. CENAIM, San Pedro de Manglaralto, Ecuador. pp. 45-52.

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FROM DISCOVERY TO COMMERCIALIZATION

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Biochemical composition and digestive enzyme activity in larvae and postlarvae of *Penaeus japonicus* during herbivorous and carnivorous feeding

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The Potential for Replacement of Live Feeds in Larval Culture

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