

The Role of Phytoplankton in the Nutrition of the  
Larvae of the Giant Freshwater Prawn  
Macrobrachium rosenbergii (De Man) (Crustacea:  
Palaemonidae)

by

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Thesis Submitted for the Degree of Master of Science

Zoology Department  
University of Cape Town  
November 1985

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## ACKNOWLEDGEMENTS

I would like to thank Dr Peter Cook for making this project possible and for his assistance, Dr Muffy Seiderer for her help in examination of my material on the Scanning Electron Microscope, Mr Daniel Garside for the Dissolved Amino Analysis of the algae exudate samples, and Mr Terry Longman for his help in making equipment on numerous occasions.

I would also like to thank Dr R K Thompson in Mauritius for her assistance, and the Camaron Hatchery Co. Ltd., for making their hatchery accessible to me.

To Sheryl Jarecki, who typed this manuscript with care and dedication, a special thank you.

Finally, I owe a great debt to the late Dr Elena Horgan, without whose friendship, and patient and valuable guidance, my work on the lipids would have been impossible. To her, I owe all my gratitude.

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ABSTRACT

The possible role of phytoplankton in the nutrition of the larvae of the prawn M. rosenbergii was investigated in this study which is conducted in two parts. In the first section of Part I, the utilization of the yolk and its lipids in the egg and first two larval stages was examined. It was found that the yolk lipids in the egg undergoes very little change during egg development and that these lipids provide for 94% of the metabolic requirements of the first stage larvae. In section two of part one, the changes in the lipids and fatty acids in larvae reared in phytoplankton rich cultures and in clear water were examined to determine whether the phytoplankton had any effect on the composition of these lipids. As no overall significant differences were found in the lipid and fatty acid compositions of larvae reared in phytoplankton rich or clear water systems, it was concluded that phytoplankton had no significant effect on the composition of lipids in M. rosenbergii larvae.

In part two of the study the uptake and fate of dissolved  $^{14}\text{C}$ -glycine was examined. The uptake of  $^{14}\text{C}$ -glucose and  $^{14}\text{C}$ -labelled algal exudate was also examined. It was found that a mechanism for the uptake of these dissolved organic substances does exist in the larvae. It was also shown, however, that the dissolved organic compounds are not of major importance as a source of energy in the nutrition of the larvae. The possible role of dissolved organic compounds is discussed.

It was concluded that phytoplankton does not play an important role as an energy source for the larvae of M. rosenbergii.

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## GENERAL INTRODUCTION

The giant Malaysian prawn, Macrobrachium rosenbergii (de Man) is a large decapod crustacean found naturally over large areas of Southeast Asia, India, Australia and areas around the South China Sea. The genus Macrobrachium includes well over one hundred species which have been described for Southeast Asia by Holthius (1950) and for the Americas by Holthius (1972).

M. rosenbergii adults are found in most of the rivers and bodies of fresh water of the far East. During the breeding season, mature females migrate downstream to estuaries where the eggs are hatched and the larvae develop. Within a few weeks, the post larvae (PL) begin to migrate back towards the fresh water where they will grow to adulthood. Excellent reviews of the biology of M. rosenbergii can be found in Ling (1969) and Wickins (1976).

Experiments on the breeding, hatching, rearing and culturing of this prawn under controlled conditions were started by Ling in 1959 at the Fisheries Research Laboratory, Penang, Malaysia as part of an FAO/Malaysian fisheries project. Successful rearing techniques for larvae and juveniles were first reported by Ling (1962).

Fujimura worked on the development of techniques for mass culture of larvae and the production of juveniles at the Anuenue Fisheries Research Center of the Hawaii Department of Land and Natural Resources in Honolulu. Once the techniques were mastered (Fujimura, 1966),

research into pond grow-out was initiated, starting with the now famous Ota's Ponds (Fujimura and Okamoto, 1970). The juvenile mass production and pond grow-out techniques developed by Fujimura are now widely accepted and used in many countries where aquaculture of M. rosenbergii is practised.

A number of characteristics make M. rosenbergii particularly attractive for aquaculture (Sandifer and Smith, 1974). Firstly, M. rosenbergii can be induced to mature, mate and spawn in captivity throughout the year and thus provides a reliable source of seed stock that can be controlled. Secondly, M. rosenbergii is hardy, omnivorous, grows rapidly and can attain a large size (up to 500gm). Thirdly, while the larvae will complete the development in brackish water only, juveniles grow to adulthood in fresh water or slightly saline conditions. This allows control and manipulation of stocks in grow-out ponds. As a result, M. rosenbergii has now been imported into Hawaii, Tahiti, the Americas, the Caribbean, Mauritius, Japan and Taiwan for aquaculture development.

The method developed by Fujimura for the mass culture of larvae and juveniles (Fujimura, 1966; Fujimura and Okamoto, 1970) uses large, non-recirculating or "static water" tanks with regular changes of water and active aeration. The cultures are supplemented with "green water", a dense mixture of phytoplankton and zooplankton. The dominant phytoplankton is usually Chlorella sp. though this varies from hatchery to hatchery.

Though practised at low prawn density (10 post larvae  $L^{-1}$ ), this system is widely used, especially in the larger prawn hatcheries (Sandifer et al., 1977). Details of this system will be given in the larval rearing section.

The use of phytoplankton has always had an important relationship with aquaculture systems, in particular with molluscan mariculture where phytoplankton is the major and most practical source of food for these filter feeders. Though crustacean larvae and post-larvae are generally less dependent on phytoplankton for nutrition, algal supplements have traditionally been used in the culture of larval penaeid prawns (Hudinaga, 1942). Recent research has shown the necessity of an algal diet for the first zoeal stages in certain penaeids (Cook and Murphy, 1969; Mock and Murphy, 1971; Meyers, 1971). The precise role of phytoplankton in the culture of M. rosenbergii, however, is unclear.

The use of algae was suggested by Fujimura (1966) and Fujimura and Okamoto (1972) as a means of increasing the efficiency of M. rosenbergii larviculture. They reported that cultures supplemented with "green water" normally resulted in an increase in larval survival and a decrease in the length of the larval cycle. Wickins (1972) reported similar results when using unialgal supplements, mainly Isochrysis galbana and Tetrasalmis suecica.

Work done by Maddox and Manzi on the larvae of M. rosenbergii at the Marine Resources Research Institute (MRRI) at Charleston, South

Carolina, has demonstrated that survival is higher and growth faster when high densities of monospecific algal cultures, especially Phaeodactylum tricornutum, are added to the larval culture (Maddox and Manzi, 1976; Manzi and Maddox, 1976). Manzi et al (1977) have shown a close positive correlation between survival of larvae and concentration of algal cells. These investigators have obtained production levels as high as 64 post larvae/litre of tank capacity, with 83% average survival, in their research hatchery.

The AQUACOP team working on Tahiti in French Polynesia have developed a culture technique which uses conical-bottom tanks, a static water system with one daily exchange of water, no phytoplankton, heavy aeration and the use of bactericides. They have produced up to 62 post larvae/litre of tank capacity (AQUACOP, 1977a; AQUACOP, 1979). Compared to the "green water" method, this technique is simpler as it involves only one trophic level, the larvae. The "green water" method depends on the quality of phytoplankton grown in special culture tanks, which fluctuates according to meteorological conditions, this placing, in a commercial hatchery, constraints on the smooth running of the system.

Research carried out in Tahiti (AQUACOP, 1977b) on larval diseases has shown that larvae cultured in the clear water system can develop an epiphytic bacterial fauna on the appendages, the gills and the setae. They also found that bacterial necrosis developed at the base of the appendages which partly or completely destroyed these. The occurrences are more serious in younger larvae and mortalities of up to 100%

resulted within 48 hours. The epiphytic bacteria disappeared immediately after antibiotic treatment, however, allowing the larvae to recover. Their results have shown, though, that M. rosenbergii larvae reared in "green water" do not have pathological problems or bacterial growths.

To date, very limited research has been done on the role played by phytoplankton in the culture of M. rosenbergii larvae. Maddox and Manzi (1977) demonstrated that, though algal cells were often ingested by larvae, there was no evidence that the larvae derived any direct nutritive benefit from the phytoplankton. Similarly, in studies with <sup>14</sup>C-labelled Tetrasalmis and Phaeodactylum, Cohen et al (1976) found that the algae were not assimilated by M. rosenbergii larvae. They concluded that algae enhance the growth of prawn larvae only indirectly by removing toxic substances such as ammonia from the rearing medium and that, where other means of waste product removal are used, the algae have no effect and may be eliminated. The MRRRI team, on the other hand, failed to demonstrate any correlation between the presence of algae and the levels of nitrate, nitrite and ammonia in the rearing water. Thus, their results indicated that "the water quality parameters measured have little, if any, relationship to the enhancement of M. rosenbergii larval culture by algal supplements" (Maddox and Manzi, 1976).

The possibility that algae provide indirect nutrition for the prawn larvae by serving as food for the Artemia nauplii fed to the larvae was also investigated (Maddox and Manzi, 1976). They found that

freshly hatched Artemia salina nauplii with ample yolk reserves are a better ration for M. rosenbergii larvae than older Artemia nauplii fed on phytoplankton.

Joseph (1977) studied the fatty acid profile of 32 day old larvae reared in different phytoplankton pure cultures. He found no correlation between the fatty acids of the larvae and those of the phytoplankton cultures used. He also found no differences in the fatty acids of the larvae, whether or not they had been reared in the presence of phytoplankton. No further work has been carried out in this field.

Thus, the role of phytoplankton in the culture of M. rosenbergii larvae remains uncertain. Maddox and Manzi (1976) suggested that algal metabolites act as growth factors for M. rosenbergii larvae, but no research has been done in this field. Sandifer et al (1977) suggested that the algae might act as a bactericidal agent. Algae are known to release antibiotic substances in water (Round, 1973). Jorgensen (1962) has shown the presence of antibiotic metabolites from three unicellular algae and Spoehr et al (1969) has shown the presence of antibacterial fatty acids released by Chlorella sp.

Certain algae are also known to produce substances which can cause a drag reduction in water similar to that produced with synthetic long-chain polymers (Hoyt, 1966). At least one of these synthetic polymers, polyethylene oxide, has been shown to beneficially affect

survival of the larvae of caridean shrimp (Palaemonetes spp.) in an unknown manner (Sandifer and Zielinsky, 1975).

In an attempt to answer some questions on the role of phytoplankton in the culture of M. rosenbergii larvae two fields of research were selected for the present study. Firstly, the lipid and fatty acid composition during the egg development and larval cycle of the prawn was examined. The study was done in two parts. In the first part the lipid and fatty acid composition of the egg during maturation was investigated whilst the second part was the study of the lipids during the larval cycle of the prawn reared in a commercial hatchery, in "green water" and in clear water.

The second major line of investigation was the study of the uptake of dissolved organic substances by the larvae of the prawn. For this purpose, larvae were reared in our facilities in the Zoology Department at the University of Cape Town.

### LARVAL REARING

The following is a description of the Methods used at the Camaron Hatchery Co. Ltd in Mauritius. Adaptations of the method for use in the rearing facilities at the University of Cape Town are also described.

Adult females with eggs are seined from freshwater grow-out ponds, brought to the hatchery and placed in brackish water until the eggs mature. Upon hatching the planktonic larvae (2,0 - 2,2 mm) are transferred to the larval rearing tanks. Here they go through eleven larval stages in 35 - 45 days and then metamorphose into benthic post-larvae (PL) which resemble miniature adults and are 0,8 - 1,2 cm long. These developmental stages have been described in detail by Ling (1967) and Uno and Kwon (1969). The PL are then conditioned to fresh water over 2 - 3 days and then transferred to the grow-out ponds. A description of the hatchery in Mauritius is given by Thompson (1980).

The young larvae are transferred to the rearing tanks at an initial density of about 40 animals/Litre. The salinity of the water is kept at about 12 - 15 ‰, pH at 7,5 - 8, and the temperature between 27° and 30°C. The water is rich in phytoplankton. The larvae are fed from the second day on with shredded tuna flesh and young Artemia nauplii. During the day, small amounts of tuna are fed at regular intervals to the larvae to avoid accumulation of uneaten food and decomposition in the water. As the larvae grow, the amount of fish is

gradually increased. A measured quantity of freshly hatched Artemia nauplii is added to the tank in the afternoon to serve as food for the night. The amount of Artemia is carefully controlled and is also gradually increased as the larvae grow.

To minimise water pollution, uneaten food material and larval wastes accumulated at the tank bottom are siphoned out daily. Partial change of water is done every second or third day when about three-quarters of the tank water is removed and replaced with fresh "green water" of similar salinity and temperature. To prevent larval loss when the water is removed from the tanks, it is siphoned slowly through a fine screen. Favourable conditions in the rearing tanks are maintained by active aeration and agitation.

The "green water" is a mixture of phytoplankton cultured in outdoor tanks in full sunlight. The dominant species in Mauritius are usually Chlorella spp. and a dinoflagellate. Species composition, however, may vary according to environmental conditions and temperature (Thompson, personal communication). A commercial fertilizer is added to the water to help algal growth. Culture tanks are used on a rotational basis and new tanks are usually seeded from old ones.

For the purpose of this study, larvae in one larval culture tank were reared to metamorphosis in the absence of phytoplankton. All the techniques described above were used but in the absence of the phytoplankton. Clear water was prepared for the exchange, as required. Two other batches of larvae were reared in "green water" using the

techniques explained above.

For the work on the uptake of dissolved organic compounds (DOC) larvae were cultured in our facilities up to the third larval stage. Techniques were similar to those outlined above and the adults were obtained from Mauritius. The phytoplankton used was usually a pure culture of Tetrasalmis suecica or Phaeodactylum tricornutum grown in our laboratory. The food given was Artemia nauplii and shredded tuna flesh.

PART I

A study of the lipids and their changes during the egg and larval development of M. rosenbergii, with particular reference to possible differences occurring in the larvae reared in different "green water" and clear water conditions.

PART IA

Yolk utilization and yolk lipid changes during the egg development and early larval stages of M. rosenbergii.

## INTRODUCTION

At spawning the eggs of M. rosenbergii are attached tightly in bundles to the first few pairs of pleiopods. The eggs are slightly oval in shape and measure about 0,6 - 0,7 mm in its long axis (Ling, 1962). The female prawn carries her eggs on her pleiopods throughout the period of incubation taking care of them until they hatch. At temperatures of 26° - 28° C incubation takes about 19 days. When the eggs are first spawned they are bright orange. After about 10 days they become lighter coloured and yellowish. The eggs progressively turn grey, their colour deepening until the 18th day of incubation, when the larvae inside the egg cases are fully developed and the colour is slate gray. Hatching takes place during the 19th - 20th day.

The early eggs consist almost entirely of innumerable minute globules of yolk. During incubation this yolk is used up as the tissues develop. The purpose of this chapter is to describe the utilisation of the yolk in the egg and early larval stages and to describe the changes in the composition of the lipids during egg development.

## MATERIALS AND METHODS

### Collection of Material

For biochemical analysis, eggs, at the various stages of development, were detached from the females in berry as required, using tweezers.

The larvae were reared in our laboratory by the method given in the section on larval rearing. Stage 1 larvae (LS1) were collected for analysis from the hatching tank shortly after these had hatched and the Stage 2 larvae (LS2) were collected from the larval culture. Two types of LS2 were collected: the first type, which still had an abundance of yolk, was collected shortly after the larvae had moulted from LS1 to LS2, and the second type more than 12 hours after moulting to LS2 when all the yolk had been used up. The larvae were not fed during these two larval stages.

#### Biochemical Analysis for Lipid Content

The eggs and larvae were washed free of sea-water with distilled water and freeze dried. Between 2 - 3 mg of freeze-dried material, for each sample, were milled and the total lipid and neutral lipid contents were determined by the methods described by Holland and Gabbott (1971) (Appendix 1). Orange eggs, yellow eggs, LS1 and LS2 larvae were analysed.

Eggs and larvae were counted, freeze-dried and weighed on a Sartorius 4503 Microbalance.

#### Analysis of Egg Lipids

The total lipids were extracted by the method of Bligh and Dyer (1959) (Appendix 2). As the yolk is uniformly distributed in the orange and yellow eggs, the whole egg was used in these two stages of

development. However, as the yolk is very compact and well defined in the grey eggs, this was removed from the egg using a micropipette. Lipid class composition was examined by thin layer chromatography (TLC) (Appendix 3). The lipid samples were methylated (Appendix 4) and analysed by gas chromatography on a Hewlett-Packard 5710A gas chromatograph using an OV73 glass capillary column for the fatty acid composition analysis (Appendix 5).

#### Photography

The eggs were mounted on slides in a drop of water for photography. The larvae were first killed in cold water then mounted on slides in a drop of water for photography. The camera used was a Wild Mka 1 model attached to a Wild M 11 compound microscope with transmitted light.

#### RESULTS

The yolk of the young eggs consist of minute globules which diminish in quantity as the eggs mature. By the time the eggs are grey and the larvae almost fully developed in the egg membrane, most of the yolk has been used up. A certain amount, however, remains in the antero-dorsal part of the larval just above the eye (Figure 1). When these larvae hatch (LS1) the eyes are sessile and some yolk is still present in the form of globules (Figure 2). After about 24 hours the LS1 larvae moult to the LS2 larvae which have stalked eyes and still retain some yolk (Figure 3). The yolk, however, is completely used up by the time the LS2 larvae are twelve hours old (Figure 4).

Dry Weights

Yellow eggs	:	60 ug/egg
LS1 larvae	:	55 ug/larva
LS2 larvae (with yolk)	:	30 ug/larva

BIOCHEMICAL CHANGES

The percentage changes in the lipid content during development from egg to larvae is shown in Table 1.

TABLE 1 Total lipid and neutral lipid composition of eggs and larvae.

	Total Lipid percent	Neutral Lipid percent	Ratio TL : NL
Eggs (Orange)	17,8	10,3	1 : 0,57
(Yellow)	11,4		
LS1 larvae	11,4	6,9	1 : 0,60
LS2 larvae	5,3	4,2	1 : 0,79

A great reduction in the total lipid content is seen as the yolk is used up in the egg and larvae.

The lipid class and fatty acid composition of the developing egg are given in Tables 2 and 3. The stages of development in the eggs used for the analyses was as follows:

A	:	2 - 3 day old	bright orange
B	:	8 day old	yellowing
C	:	15 day old	grey
D	:	18 - 19 day old	grey with eye spots

**TABLE 2** Percentage composition of lipid classes in M. rosenbergii eggs

Sample	PLP	F.Alc	FFA	TG	X	WE
A	17	2	15	53	3	10
B	17	2	13	51	3	14
C	19	1	13	51	6	10
D	21	1	12	55	2	9

All values given as a percentage of the total lipid in the sample. PLP: Polar lipid and pigments; FFA: Free fatty acids; F.Alc: Free fatty alcohols; TG: Triacylglycerols; X: unknown compound; WE: Wax esters.

TABLE 3 Percentage composition of yolk fatty acids in eggs.

Fatty Acids	SAMPLE			
	A	B	C	D
14 : 0	1,0	1,2	1,3	1,5
16 : 0	23,1	24,2	24,7	25,7
16 : 1 (W7 + W9)	8,3	7,2	8,2	7,6
17 : 0	---	0,4	---	---
17 : 1	1,0	1,1	1,0	1,0
18 : 0	6,1	5,3	6,1	6,6
18 : 1 (W7 + W9)	41,3	43,6	41,4	40,8
18 : 2 W6	12,6	11,6	12,1	11,0
18 : 4 W3	0,7	2,1	0,4	---
20 : 1	0,9	0,8	0,7	1,0
20 : 4 W6	1,1	0,4	0,6	1,0
20 : 5 W3	2,5	1,3	1,5	2,4
22 : 1	---	0,3	1,5	---
22 : 5 W3	---	0,1	0,5	---
22 : 6 W3	1,4	0,4	---	1,4

### DISCUSSION

Moller (1978) has shown that LS1 larvae do not feed. The feeding appendages are folded against the thorax and appear to have little function. LS1 larvae moult to LS2 about 24 hours after hatching, at which stage the appendages used for feeding become readily extended for grasping food particles. The results in this paper show that a certain amount of yolk is carried by the LS1 larvae from the egg. It is also shown that some yolk is still found in LS2 larvae during the first few hours, but this later disappears.

It is suggested that the yolk in the LS1 larvae is used as an energy reserve until the larvae have moulted to LS2 and at which stage they can start feeding. It is also suggested that the yolk found in the

early LS2 larvae is necessary to maintain the energy requirements of these larvae until they have built up reserves.

Yolk utilization in marine crustacean eggs has been described by Pandian (1969, 1970a, 1970b) and reviewed by Holland (1978). The proteinaceous yolk of crustaceans is thought to be made up of aggregates of lipoproteins termed lipovitellins (Holland, 1978). The lipid fraction forms approximately 30% of the proteinaceous yolk in many decapod eggs and contains carotenoid pigments which give the eggs their distinctive colour (Wallace et al, 1963). In the present case, the total lipid content of the yolk in early M. rosenbergii eggs is 17,8%, somewhat higher than the value of 12,3% for Macrobrachium idella yolk given by Vijayaraghavan and Easterton (1974). By hatching time the total lipid content of LS1 larvae is 11,4%, which represents a 36% utilization of lipids during egg incubation, which is very similar to the results given by Pandian (1970b) for Crangon crangon, Euragurus beruhardus and Homarus gamarus.

The dry weight of eggs was 60 ug/egg, and the dry weight of LS1 and LS2 larvae was 55 ug and 30 ug/larva respectively. The amount of lipid available in the egg is, therefore, 10,68 ug, which has a calorific value of about 0,4225 joules, using the calorific value of lipid given by Crisp (1971) as approximately 39,558 joules/mg lipid. Since LS1 larvae have a lipid content of 11,4% of their weight, the lipid available per larvae is 6,27 ug having a calorific value of 0,24803 joules. The LS2 larvae have only 5,2% of their weight in lipid weighing 1,56 ug and with a calorific value of 0,06171 joules

per larva. The calorific value of yolk utilized by LS1 larvae is 0,18632 joules. If lipids represent the major respiratory reserves of crustacean eggs and larvae as suggested by Holland (1978) and Pandian (1970b), the lipid loss in LS1 should be accounted for by respiratory losses of LS1 larvae since these do not feed. Respiration values given for LS1 larvae by Nelson et al (1978) are 3,1546 joules/ug/day. The calorific value of respiration/day for one larva weighing 55ug is, therefore, 0,17405 joules. Since LS1 larvae remain in that stage for about 24 hours, the energy requirements are very similar to the calorific equivalent of 0,18631 joules of lipid used up during LS1. This suggests that the lipid in the yolk provide the bulk (94%) of the energy requirements for the S1 larvae.

The lipid class composition of the yolk in the egg does not change much during development with the exception, perhaps, of the polar lipids and pigments which appear to increase slightly. The triacylglycerols form the major part of the lipids in the yolk.

Pandian (1967, 1970a, 1970b) has shown that the lipid fraction of the yolk in the egg of the shrimp Crangon crangon fulfills 75% of its metabolic requirement, 87,7% in the lobsters Homarus gammarus and 87,9% in the isopod Ligia oceanica and that the protein component remains relatively unchanged. It has been clearly demonstrated, in the present study, that the lipid fraction of the yolk fulfills the bulk of the energy requirements of the non-feeding LS1 prawn larva. One can assume that it would have a similar function in the egg. The

above results also show that during egg development components of the yolk lipid are used up uniformly.

One can, therefore, conclude that the lipid fraction of the yolk provides the bulk of the energy supply in the egg and non-feeding larval stage of M. rosenbergii.

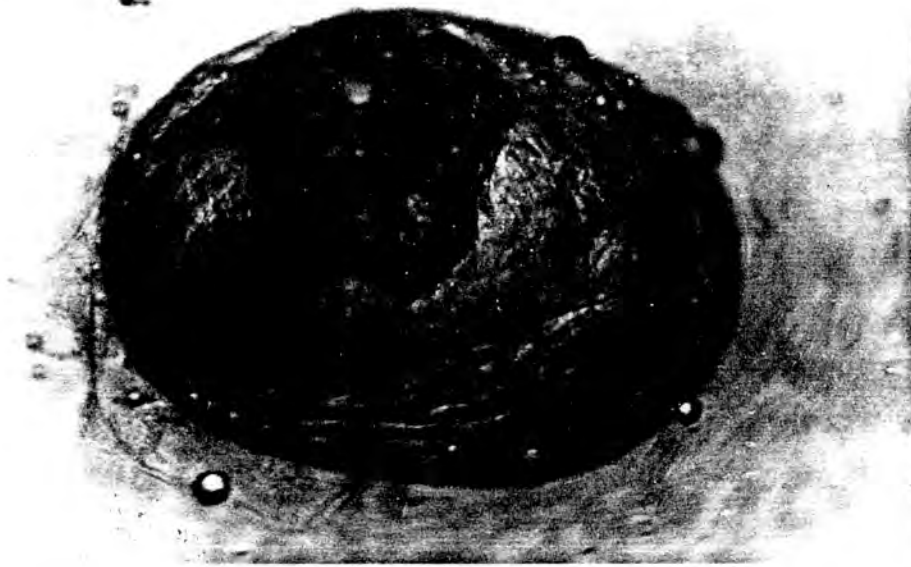


FIGURE 1: 18 day old egg showing yolk granules and the eye.

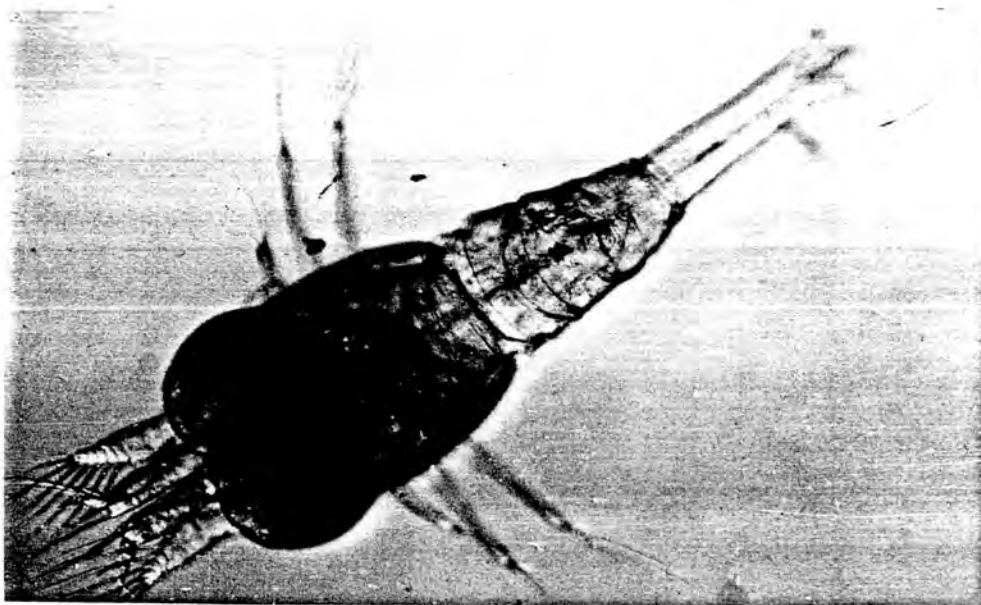


FIGURE 2: Freshly hatched LS1 larva showing yolk granules behind the sessile eyes.

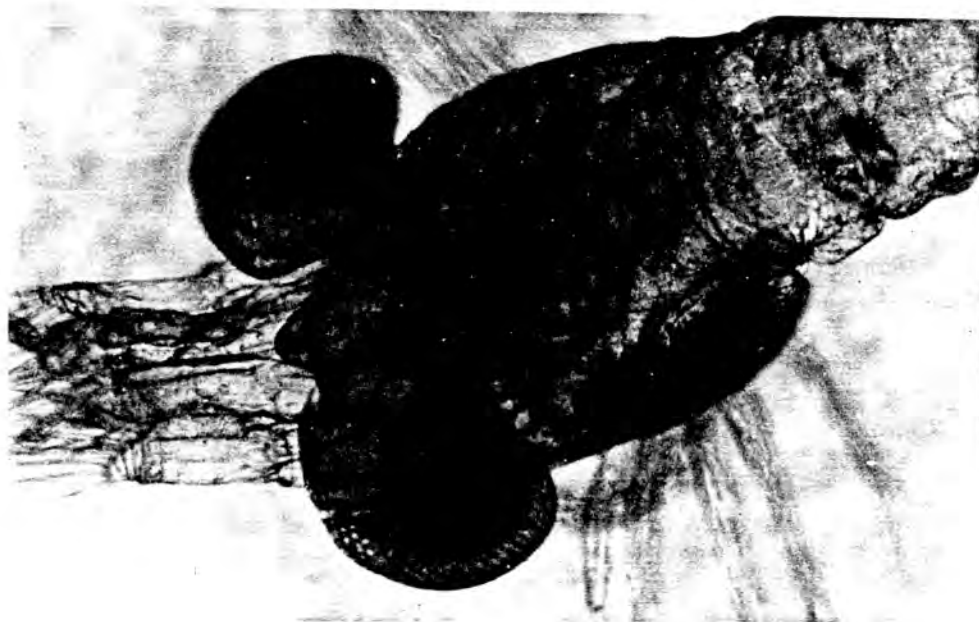


FIGURE 3: LS2 larvae shortly after moult from LS1. Yolked granules are visible behind the stalked eyes.

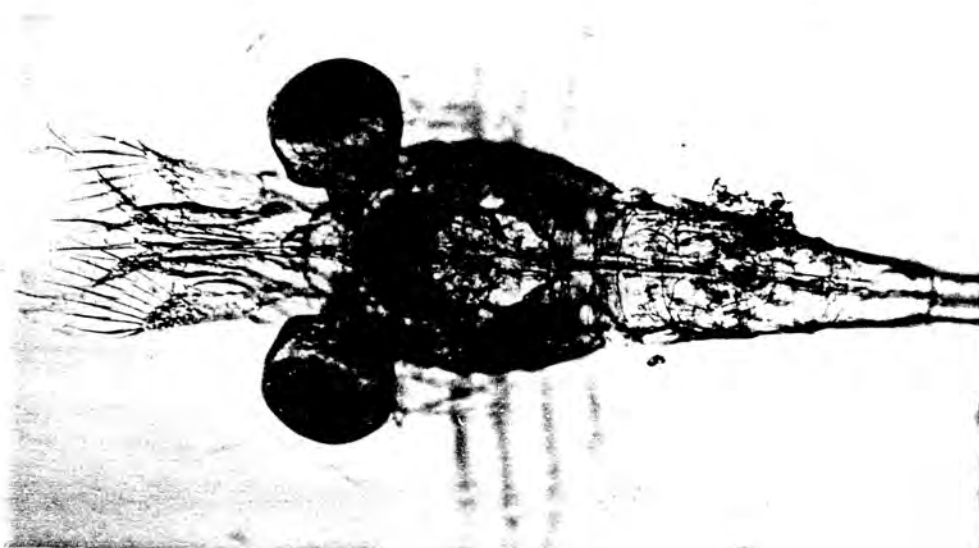


FIGURE 4: 12 hours LS2 Larva showing the absence of yolk.

PART IB

A study of the changes in the lipids and fatty acids during the development cycle of M. rosenbergii larvae reared in "green water" and in clear water.

## INTRODUCTION

Lipids are the second largest biochemical fraction after proteins in juveniles of M. rosenbergii (Choo Poh Sze, 1973). Probably one of the most important factors influencing both the amount and the composition of lipid in marine animals is diet (Morris and Culkin, 1976). Langdon and Waldock (1981) showed that the fatty acid composition of the triacylglycerols of the spat of the oyster Crassostrea gigas reflected that of the diet. Kayama et al (1980) showed a clear relationship between diet and fatty acid composition in the juveniles of the prawn Penaeus japonicus. They also demonstrated that the fatty acid composition in the non-polar or neutral lipids followed a similar pattern to that of the polar lipids. Sandifer and Joseph (1976) showed that the fatty acid composition of juvenile M. rosenbergii changed with that of the diet. Feeding studies with crustaceans using various types of plant or animal lipids and purified test diets containing pure fatty acids and sterols have suggested a requirement for fatty acids of the linolenic or  $\omega 3$  series (Castell and Boghen, 1979; Guary et al, 1976; Kanazawa et al, 1977; Kayama et al, 1980; and Sandifer and Joseph, 1976).

The aim of the present study was to examine the changes occurring in the fatty acid and lipid class compositions of the larvae of M. rosenbergii during the course of their development, with particular reference to differences occurring between larvae reared in "green water" and those reared in clear water.

## MATERIALS AND METHODS

### Animals

The animals were obtained from the Camaron Hatchery Co. Ltd., in Mauritius. Each larval culture tank at that hatchery holds 24m<sup>3</sup> of water and can produce in excess of 120 000 PL per cycle. The tanks are initially stocked with hatchlings from 30 - 40 females. The advantage of this for the present study is that it reduced genetic variation in the larvae. However, the tanks sampled were not all stocked with females from the same area, so initial differences between tanks could be due to the different brood stocks. Larvae reared in "green water" were sampled from two tanks (Cultures 1 and 2 or C1 and C2). Larvae reared in the absence of phytoplankton were sampled from one tank (C3). All three tanks were managed in the same way described in the larval culture section.

On sampling, the larvae were sorted into larval stages (LS), rinsed in fresh water and frozen in sealed tubes at -20° C for storage until they were needed for lipid extraction.

### Lipid Analysis

Total lipids were extracted by the method of Bligh and Dyer (1959) (Appendix 2). Tissue was homogenised and lipids extracted under nitrogen. The composition of lipid classes was determined by thin layer chromatography (TLC) on 0,25 mm thick, 20 x 20 cm Merck silica gel plates (Appendix 3). Lipids from each larval stage were banded on

the plates using a CAMAG Linomat III spot applicator. The plates were developed with a mixture of petroleum ether : diethyl ether : acetic acid (90:10:1 v/v/v). The plates were developed twice. The lipids were visualised by spraying with 40% sulphuric acid and charring at 180° C for about 15 minutes. The lipids were identified by comparison with known standards. The resulting bands were quantified using a Vitatron TDL Universal Densitometer.

To prepare the lipids for fatty acid analysis by gas chromatography aliquots of total lipid extract were methylated to produce fatty acid methyl esters using Boran fluoride-methanol (Morrison and Smith, 1964) (Appendix 4). Gas chromatographic analysis for fatty acid composition was done on a Hewlett Packard 5710A Gas Chromatograph and an H/P 18740 A capillary column control. The column used was a OV73 glass capillary column (Appendix 5). Fatty acids were identified by comparison with the retention times of known fatty acid methyl esters and their area percentages were calculated on a Hewlett-Packard 3352A Laboratory Data Processor.

Subsamples of larvae were counted, freeze-dried and weighed for each larval stage in the three cultures. Their total lipids were extracted by the method of Bligh and Dyer (1959) and evaporated to dryness under a flow of nitrogen. The lipid extracts were then weighed. All weighings were done on a Sartorius 4503 Microbalance. Growth curves and percentage lipid contents for each larval stage could thus be determined. All lipid values are calculated on a milligram lipid per gram tissue dry weight basis.

## RESULTS

### Larval Growth

Figure 5 gives the growth curves for the three cultures (C1, C2 and C3). No significant differences exist between the three cultures except for the PL of C2 which are smaller than those of C1 and C3. It is possible that when the PL of C1 and C3 were sampled, they were slightly older than the PL of C2.

### Lipid Content and Lipid Class Composition

The total lipid content (TL) of the larvae in C1, C2 and C3 are shown in Figure 6. In all three cultures, there is a sharp drop in TL from 190 ug/g tissue dry weight at larval stage 1 (LS1) to about 75.0 ug/g dry weight at LS3. This is followed by an increase at LS4 in the three cultures but this drops back to about 75 ug/g tissue at LS5 in C1 and C2 after which the level of lipid remains fairly constant right through to the post larval stages. In C3, the lipid content after increasing at LS4 remains high up to LS6 where it drops to 82 ug/g dry weight at LS7 at which point it maintains a level similar to those of C1 and C2 for the remainder of the cycle.

The neutral lipid (NL) pattern (Figure 7) shows a similar trend to that of the total lipid in the three cultures (Figure 6). There are no significant differences in lipid contents in C1, C2 and C3, except for that of LS5 and LS6 of C3 which are higher than in the corresponding stages of C1 and C2.

Figures 8 to 13 show the changes in the total lipid percentage compositions of the phospholipids (or polar lipids) and pigments (PLP), the diacylglycerols (DG), the cholesterol (CH), the free fatty alcohols (FA), the free fatty acids (FFA) and the triacylglycerols (TG). Figures 15 to 20 show the changes in content of these lipids during the larval cycles in relation to the tissue dry weight.

The content of the phospholipids and pigments (PLP) in the tissues are significantly different at most of the larval stages in the three cultures, except for LS7 - LS11 in C2 and C3. The change in PLP content in C2 and C3, however show similar trends, both reducing from about 55 ug/gm and 37 ug/gm at LS1 to 30 ug/gm and 18 ug/gm at PL, respectively. In C1 there is no general decrease of the PLP, the amounts at LS1 and PL being similar at about 25 ug/gm (Figure 15). The percentage composition of the PLP in the total lipid, however, shows different trends which appear to be unrelated to the tissue content of PLP. In C1 and C2 there is a gradual increase in the percentage content of the PLP from about 13% and 30% respectively. In the C3 larvae, though there are major fluctuations during the larval cycle, no dominant trend emerges.

There are no major significant differences in the diacylglycerol (DG) percentage composition and tissue content in cultures C1, C2 and C3 (Figures 9 and 16). Though there are significant differences at individual larval stages, no trends are evident except in C2 when the DG content shows a gradual reduction from about 1 ug/g at LS1 to 0,4 ug/g at the post-larval stage.

Though there are differences in the cholesterol (CH) percentage composition of the lipids and in the cholesterol content of the tissues (Figures 10 and 17), there are no significant differences in the cholesterol patterns during the complete cycle in cultures C1, C2 and C3. The percentage composition in the three cultures show a twofold increase from about 12% of the total lipid (TL) at LS1 to about 22% of the TL at the post-larval stage (Figure 10). In all three cultures there is a general reduction of cholesterol content in the tissue from about 20 ug/g dry weight at LS1 to about 17 ug/g in the post-larvae.

The free fatty alcohols (FA) of cultures C1 and C2, when expressed as a percentage of total lipid show a similar pattern of net increase from 4% and 8% at LS1 to about 13% and 19% of the TL at the post larvae, respectively. In both these cultures there is a drop in the percent content of the FA during second half of the larval cycle but this increases again at LS11. In the C3 larvae, the FA increases from 6% at LS1 to 28% at LS6 followed by a gradual decrease to about 14% in the post-larvae which represents a net increase of 8% (Figure 11). When the FA are calculated in relation to the dry weight of tissue the levels of FA in three cultures remain fairly constant at between 8 and 12 ug/g throughout the larval cycles, with the exception of LS5 and LS6 in C3 where the FA increase to 30 ug/g of dry tissue weight. By LS8, however, the level has dropped back to 12 ug/g (Figure 18).

There are no significant differences in the free fatty acid (FFA) profiles during the larval cycles in C1, C2 and C3. The percentage composition of the FFA remains fairly constant at about 10% with

individual fluctuations between 5 and 20% (Figure 12). When the fatty acids are expressed as a portion of the dry tissue weight, they decrease from about 20 ug/g at LS1 to about 7 ug/g at LS3 in C1, C2 and C3. This is followed by an increase in LS4 to between 15 ug/g and 20 ug/g after which there is a general decrease to about 7 ug/g with the exception of LS11 and the PL of C1 where the FFA increases to 20 ug/g (Figure 19).

The percentage composition of the triacylglycerols (TG) have very similar patterns in C1, C2 and C3. The proportion of TG in the total lipid drops sharply from between 32 - 38% in LS1 to between 4% and 8% in LS3 after which the composition remains at about the same levels to the PL stage (Figure 13). When the TG are calculated as a value of the dry tissue weight, the pattern is similar to that of the percentage compositions (Figure 20). After a rapid drop from an initial level of 60 to 70 ug/g dry weight at LS1 to about 7 ug/g at LS3 the TG remain at level for the rest of the cycle in all three cases.

Figure 14 shows the percentage composition of an unknown compound which has been labelled as and is assumed to be part of the total lipid fraction. On the TLC plates the substance runs just above the triacylglycerols at a level similar to that of the alkyldiacylglycerols. Sargent and Falk-Petersen (1981) have reported the presence of a substance occurring in the same position in the krill Meganyctiphanes norvegica where it formed up to 11% of the total lipid. They failed to identify the compound.

In the present case, comparison with known standards failed to identify the substance, as also did attempts to identify it by mass spectrography.

The percentage content of unknown X in the total lipid remains fairly constant at about 10% for all larval stages in C2 and C3 except for a peak at 20% in LS10 in C2 and an increase to 21% in LS11, LS12 and PL in C3. In C1, the pattern is quite different. At LS1, the level is similar to that of C2 and C3, but there is a rapid increase to 52% at LS3 and LS4 which is followed by a gradual decline, except for a peak at LS8, to 8% at LS11 and PL.

When X is calculated as a value of the dry tissue weight (Figure 21), it shows almost no difference in the C2 and C3 cycles where it maintained a fairly constant level ranging between 10 and 18 ug/gm dry weight. Again, in C1, the pattern is very different with X starting at 55 ug/gm dry weight at LS1 and progressively dropping to 8 ug/g dry weight at LS11 after undergoing a series of peaks and troughs.

There seems to be no possible correlation between the contents of X and the presence of phytoplankton as in C1 and C2 and its absence as in C3.

Table 4 gives the percentage composition of the lipid classes of the phytoplankton used in cultures C1 and C2. There are major differences in the lipids of these two algae. In C1 the phospholipids and

pigments make up only 10,3% of the total lipids, whereas in C2 these make up 50,5% of the total lipids.

An unidentified sterol is present in both algal cultures, but C1 has 8% and C2 has only 4,3% of this sterol making up the total lipid. The fatty alcohols in both cultures are similar in content with 12,0% and 14,3% for C1 and C2 respectively.

C1 phytoplankton lipid has 15,6% of free fatty acids whereas the C2 phytoplankton has only 5,2%, and C1 has 10,3% as triacylglycerols where C2 has only 2,8% of its lipids as triacylglycerols.

The unknown compound X, on the contrary, forms only 8,0% of the total lipids in C1 phytoplankton and 21,6% of the total lipids in the C2 phytoplankton.

Cholesterol esters are present in the two cultures, forming 6,6% of the lipids in C1 algae and 1,3% in C2 algae. The cholesterol esters are either totally absent or present in very small traces in the larvae of the three cultures C1, C2 and C3.

#### Fatty Acid Composition

Tables 5, 6 and 7 list the fatty acid profiles of the larvae from LS1 to PL in cultures C1, C2 and C3. Tables 2 and 3 also indicate the fatty acid profile of the phytoplankton used in C1 and C2. As with the lipid class compositions the fatty acids in the three cultures

start at different values at LS1 and show individual differences during development. A number of trends are found to be common to the three cycles, however.

The total saturated acids decrease from an average of 41% of the total fatty acids at LS1 to about 32% at LS3 after which the levels remain fairly constant to the post larval stages (Figure 22). At the level of individual acids, this pattern is found in the 14:0 and the 16:0 fatty acids which both show a rapid decrease from LS1 to LS3 after which the levels remain fairly constant. The 14:0 acid reduces from about 4% of total fatty acids at LS1 to about 1% at post larvae and the 16:0 reduces from about 30% at LS1 to about 15% at PL. The same general values are found in all three cultures. The 18:0 fatty acids, on the other hand increases from about 7.5% in LS1 to about 10% in LS2 after which its level remains fairly constant.

The total mono-unsaturated acids, i.e. the fatty acids with a simple double bond, maintain a fairly constant level at around 40% of the total fatty acids in the three cultures (Figure 23). The combined 16:1 W9 and 16:1 W7 acids, however, decrease from about 7% in LS1 to about 3% in the PL. The 18:1 W7 acid, on the other hand, increases in C1, C2 and C3 from about 6% in LS1 to between 8 and 9% in the PL. All the other mono-unsaturated acids, of which 18:1 W9 is the most abundant, show very little change in amount.

The total polyunsaturated fatty acids, or the acids with more than one double bond or unsaturation, have a profile opposite to that of the

saturated acids (Figure 24). These acids increase from an average value of 20% in LSI to about 28% in the PL. This is particularly noticeable in the two linolenic acids 20:5 W3 and 22:6 W3. 20:5 W3 increases from about 6% in LSI to about 11% in the PL and 22:6 W3 increases from about 5% in LSI to about 10% in the PL. The linoleic acid 18:2 W6, on the other hand decreases from about 6% in LSI to about 3% in the PL. These trends are common to all three cultures. The polyunsaturated acids, of all three fatty acid groups have, however, the widest fluctuations between cultures.

The fatty acid compositions of the phytoplankton used in cultures C1 and C2 are shown in Tables 5 and 6. The two algal cultures have great differences in polyunsaturated acids. In C1 phytoplankton 16:4 is absent but makes up 4,2% of the fatty acids in the C2 phytoplankton. The 18:2 W6 acid is 12,25% of the total acids in the C1 algae, but make up only 5,17% of the acids in the C2 algae. 20:5 W3, which is so abundant in the larvae, is only 0,51% in C1 algae and 4,36% in the C2 algae. 22:6 W3 is absent in both algal cultures. The saturated and mono-unsaturated acids are very similar in the two algae.

## DISCUSSION

Cultures C1 and C2, which were both "green water" cultures were not run concurrently. Thus, the "green water" used in these two cultures were from different sources and probably had different algal compositions. Though no attempt has been made to identify the phytoplankton, C1 algae consisted of a mixture of a dinoflagellate and Chlorella spp (Thompson, pers comm) and C2 algae was mainly an unidentified dinoflagellate. The differences in phytoplankton types was confirmed by the lipid class differences (Table 4) and by the fatty acid composition differences (Tables 5 and 6).

Though the lipid classes in the three cultures show a fairly wide range of individual variation, the trends observed are basically similar. Occasionally, as is the case with the unknown X (Figures 14 and 21), cultures C2 and C3 show very similar profiles, but C1 is very different from both. Interestingly, the unknown X in C1 constituted only 8% of the phytoplankton lipid but made up to 53% of the larval lipids whilst in C2 it constituted 21,6% of the algal lipid but made up 10% of the larval lipids. There seems to be no dietary relationship between the levels of the unknown in the phytoplankton, or its absence from the culture medium (as in C3) and its level in the larvae.

Similarly, the fatty alcohols in the larvae show significant differences in their individual levels in the three cultures but these

seem to bear no relationship to the level of that lipid in the phytoplankton of C1 and C2 and the absence of phytoplankton in C3.

In general, the different lipid classes followed similar trends which seemed to bear little or no relationship with the levels of these lipids in the algae or to the presence or absence of phytoplankton in the larval culture.

Though the 16:4 W1 acid is absent from the C1 phytoplankton and makes up 4,2% of the fatty acids in the C2 phytoplankton, the levels of this acid are similar in the larvae at C1, C2 and C3. Similarly, where 18:2 W6 has a level of 12,25% in the C1 phytoplankton and 5,17% in the C2 phytoplankton, the level of this acid in the larvae from the three cultures is similar, varying between 3% and 4%, with no apparent relationship to the level of this acid in the phytoplankton (C1 and C2) or its absence (C3).

The long chain 20:5 W3 and 22:6 W3 acids are reported to be essential for normal growth and development and to be diet dependent in the prawn Penaeus japonicus (Jones et al, 1979; Kanazawa et al, 1978), for juvenile Macrobrachium rosenbergii (Sandifer and Joseph, 1976) and for the oyster Crassostrea gigas (Langdon and Waldock, 1981). The levels of 20:5 W3 in the C1 phytoplankton is only 0,51% and is 4,36% in the C2 phytoplankton, yet, the level of this acid in the larvae of the three cultures is the same at about 10-11%. The other long chain acid 22:6 W3 is absent in both C1 and C2 phytoplankton but has a high level of 9 - 11% in the larvae of C1, C2 and C3.

It seems, therefore, that the level of these two fatty acids in the phytoplankton, or its absence from the culture medium, as in C3 does not significantly affect their levels in the larvae. Since these acids have been shown to be diet dependent, the larvae must, therefore, obtain them from another source, such as the Artemia or the fish that is fed to the larvae.

Finally, as was shown in Figures 22, 23 and 24, the levels of total saturated acids, total mono-unsaturated and total polyunsaturated acids were very similar in the three cultures, with no apparent differences in those where phytoplankton was present (C1 and C2) and that one where phytoplankton was absent (C3).

One can, therefore, conclude that there are no significant differences in the levels of lipids and fatty acids in the larvae of M. rosenbergii reared in different phytoplankton cultures or in the absence of a phytoplankton. If phytoplankton has any nutritional value to the larvae during their growth cycle, then the results of these experiments indicate that its nutritional role is not reflected in the lipids.

TABLE 4

Lipid Class Compositions of Phytoplankton used in Cultures C1 and C2.

	<u>C1</u>	<u>C2</u>
Phospholipids and Pigments	10.3	50.5
Diacylglycerols	1.7	-
Sterols	8.0	4.3
Fatty Alcohols	12.0	14.3
Fatty Acids	15.6	5.2
Triacylglycerides	10.3	2.8
Unknown	8.0	21.6
Cholesterol Ester	6.6	1.3

TABLE 5.  
FATTY ACID COMPOSITION OF LARVAE AND PHYTOPLANKTON IN CULTURE 1

LARVAL STAGES	1	2	3	4	5	6	7	8	9	10	11	PL	Algae
FATTY ACIDS %													
C12 : 0	0.25	0.12	—	—	0.12	0.18	0.09	0.08	—	—	—	—	0.38
C13 : 0	—	—	—	—	—	—	—	—	—	—	—	—	—
C14 : 0	4.69	1.56	1.34	1.24	1.55	2.13	2.36	1.79	1.45	1.67	1.78	1.47	1.01
C15 : 0A	0.14	—	—	0.61	0.73	0.54	0.54	0.96	0.70	0.50	0.49	0.39	—
C15 : 0	0.56	0.39	0.28	0.44	0.48	0.45	0.41	0.40	0.33	—	0.29	0.33	—
C16 : 0	29.90	21.47	21.27	16.87	17.55	17.50	17.75	15.20	15.89	16.83	17.93	17.61	33.15
C17 : 0A	0.19	0.21	0.27	0.50	0.75	0.47	0.47	0.72	0.62	0.30	0.51	0.53	—
C17 : 0 CYCL	0.45	0.33	0.32	0.91	0.74	0.64	0.62	0.87	0.74	0.41	0.54	0.57	—
C17 : 0	0.61	0.56	0.81	0.88	1.21	0.97	0.97	1.05	1.12	1.02	0.96	1.22	0.38
C18 : 0	7.30	8.12	11.00	9.18	9.79	8.97	8.81	8.65	9.74	10.65	10.14	10.88	3.68
C19 : 0	0.14	—	0.08	—	0.09	0.08	0.10	0.09	—	—	0.10	—	—
C20 : 0	0.47	0.35	0.38	0.40	0.34	0.32	0.30	0.27	0.17	0.32	0.20	0.17	—
C22 : 0	—	0.17	0.33	—	—	—	—	0.34	0.19	—	0.22	—	0.70
C24 : 0	—	—	0.18	—	—	0.08	—	0.17	0.37	—	—	—	—
TOTAL SATURATED %	44.70	36.29	36.26	31.03	33.35	32.31	32.42	30.61	31.32	31.70	32.79	33.17	39.31
C14 : 1	—	—	—	—	—	—	—	—	—	—	—	—	—
C16 : 1 W7+W9	7.02	4.92	2.44	5.41	4.20	4.40	4.36	5.33	3.78	3.11	2.98	2.91	1.49
C18 : 1 W9	22.38	23.18	16.97	24.50	21.66	18.85	18.57	26.43	26.28	24.14	25.85	20.11	35.15
C18 : 1 W7	5.86	7.01	7.87	10.11	10.02	8.68	8.59	10.71	10.78	10.05	7.92	9.95	2.67
C20 : 1	1.27	0.66	0.98	1.41	1.54	1.59	1.54	1.57	0.93	2.44	2.08	1.21	1.27
C22 : 1 W11	1.06	0.14	0.58	0.37	—	1.08	0.31	0.77	0.29	1.61	1.28	1.78	0.41
C22 : 1 W8	0.09	0.13	—	0.45	—	—	2.60	—	—	—	—	—	—
C22 : 1 W7	0.05	—	—	—	—	—	—	—	—	—	—	—	—
C24 : 11	—	—	0.15	—	—	—	—	—	—	—	—	—	—
TOTAL MONO-UNSATURATED %	37.73	36.04	28.99	42.25	38.50	36.27	35.97	44.81	42.06	42.74	40.11	35.96	41.40
C16 : 4 W1	0.16	—	—	—	0.35	0.35	0.26	0.42	0.29	—	0.39	0.31	—
C16 : 3	0.14	—	—	0.31	0.06	0.15	—	0.10	—	—	—	—	1.73
C16 : 2	—	—	—	—	—	—	—	—	—	—	—	—	—
C18 : 4 W3	0.51	0.15	0.39	1.12	0.68	0.76	0.77	0.76	0.68	1.01	0.92	0.57	4.43
C18 : 3 W6	—	—	—	—	—	—	—	—	—	—	—	—	—
C18 : 2 W6	5.13	5.58	4.48	5.10	3.94	3.22	3.15	4.71	4.53	4.26	3.93	3.70	12.25
C20 : 4 W6	1.64	7.36	5.62	3.57	2.98	2.73	2.60	2.11	2.70	2.28	1.51	2.55	—
C20 : 5 W3	5.40	9.32	14.40	11.00	11.46	12.06	12.65	9.56	11.64	10.08	10.17	11.99	0.51
C20 : 4 W3	0.15	0.11	0.15	0.41	0.39	0.37	0.41	0.47	0.52	—	0.37	0.31	—
C21 : 5	0.09	0.10	0.26	0.24	0.21	0.52	0.15	—	—	—	—	—	—
C22 : 6 W3	4.26	4.69	8.59	4.55	6.75	9.22	11.91	5.73	5.97	7.94	9.14	11.17	—
C22 : 5 W3	0.36	0.24	0.32	—	0.31	0.43	0.49	0.24	—	—	0.25	0.26	0.40
TOTAL POLY-UNSATURATED %	17.84	27.55	34.21	26.30	27.13	29.81	32.39	24.10	26.33	25.57	26.68	30.86	19.32

TABLE 6.  
FATTY ACID COMPOSITION OF LARVAE AND PHYTOPLANKTON IN CULTURE 2.

LARVAL STAGES	1	2	3	4	5	8	7	8	9	11	PL		Algae
FATTY ACIDS %													
C12 : 0	0,52	0,22	0,36	----	0,15	0,13	0,12	0,07	----	0,07	----	----	0,32
C13 : 0	----	----	----	----	----	----	----	----	----	----	----	----	----
C14 : 0	3,51	1,19	0,85	1,83	1,02	0,91	0,97	0,71	----	0,91	0,76	----	----
C15 : 0A	----	----	----	0,62	----	----	----	0,55	0,63	----	----	----	----
C15 : 0	0,57	----	----	----	----	----	----	0,43	----	----	----	----	----
C16 : 0	23,20	21,98	19,13	17,02	17,50	16,85	14,88	14,35	14,58	14,99	14,53	----	34,37
C17 : 0A	----	----	----	----	----	----	----	0,53	----	----	----	----	----
C17 : 0 CYCL	----	----	----	1,28	1,47	1,40	----	0,88	0,44	1,61	1,07	----	----
C18 : 0	0,71	0,98	0,79	0,92	1,42	1,50	1,22	1,13	0,89	1,01	1,15	----	2,30
C18 : 0	8,55	11,75	9,13	10,22	12,51	11,46	9,55	11,33	9,51	10,57	12,30	----	3,82
C19 : 0	----	----	----	----	----	----	----	----	----	----	----	----	----
C20 : 0	0,64	0,45	0,45	0,48	----	----	0,17	----	----	0,47	0,38	----	----
C22 : 0	0,47	0,56	0,34	0,33	0,33	----	----	----	0,36	0,36	0,26	----	0,80
C24 : 0	0,36	0,26	0,24	----	----	0,06	0,08	----	0,59	----	----	----	----
TOTAL SATURATED %	38,53	37,39	31,29	32,70	34,40	32,85	26,99	29,98	27,00	29,92	30,45	----	42,61
C14 : 1	----	----	----	----	----	----	----	----	----	----	----	----	----
C16 : 1 W7+W9	6,48	4,42	4,76	4,57	4,33	3,68	4,49	3,59	3,42	3,22	3,57	----	----
C18 : 11 W9	23,93	23,29	24,31	29,62	20,98	20,38	21,31	26,01	29,19	24,00	23,90	----	33,07
C18 : 1 W7	5,84	7,64	6,71	8,29	8,82	7,06	7,16	6,61	7,54	9,87	7,85	----	2,77
C20 : 1	1,50	1,15	0,67	0,80	0,90	1,09	1,35	1,65	2,07	1,83	1,50	----	1,89
C22 : 1 W11	0,69	0,26	0,56	0,16	0,18	0,42	0,09	0,20	0,67	1,46	1,30	----	0,43
C22 : 1 W9	----	----	----	----	----	----	----	----	1,31	----	----	----	----
C22 : 1 W7	----	----	----	----	----	----	----	----	----	----	----	----	----
C24 : 1	0,06	----	----	0,14	0,05	----	----	----	----	----	----	----	----
TOTAL MONO-UNSATURATED %	38,44	36,76	37,01	43,58	33,26	32,63	43,40	40,06	44,12	40,18	38,12	----	37,18
C16 : 4 W1	----	----	0,33	----	----	----	0,39	----	----	----	----	----	4,20
C16 : 3	----	----	----	0,31	----	----	----	0,28	0,38	0,47	----	----	----
C16 : 2	----	----	0,28	----	----	----	----	----	----	----	----	----	----
C18 : 4 W3	----	----	0,46	1,02	----	----	----	0,29	0,61	1,08	0,76	----	4,54
C18 : 3 W6	----	----	----	----	----	----	----	----	----	----	----	----	----
C18 : 2 W8	6,31	4,38	4,20	4,24	4,13	3,08	3,70	3,08	3,18	2,14	3,48	----	5,17
C20 : 4 W6	3,79	4,03	3,34	2,84	4,15	4,22	5,37	3,99	3,51	3,40	2,94	----	----
C20 : 5 W3	7,14	9,37	9,66	9,36	11,85	14,45	18,20	11,22	10,95	12,00	11,21	----	4,36
C20 : 4 W3	----	----	----	----	0,25	----	----	0,22	0,44	----	----	----	----
C21 : 5	----	0,48	0,30	0,29	0,41	0,67	0,21	0,60	0,70	----	0,40	----	----
C22 : 8 W3	5,37	5,00	5,44	5,10	9,81	12,87	9,22	10,23	7,92	10,78	11,13	----	----
C22 : 5 W3	0,74	0,14	----	----	0,28	----	0,55	0,44	0,33	0,43	0,42	----	----
TOTAL POLYUNSATURATED	23,35	23,40	24,01	23,16	30,88	35,29	37,64	30,33	28,00	29,83	30,32	----	18,27

TABLE 7.  
FATTY ACID COMPOSITION OF LARVAE IN CULTURE 3.

LARVAL STAGES	1	2	3	4	5	6	7	8	9	10	11	PL	
FATTY ACIDS %													
C12 : 0	0.45	0.79	---	0.22	---	0.11	0.08	0.06	0.10	0.10	---	0.07	
C13 : 0	---	---	---	---	---	---	---	---	---	---	---	---	
C14 : 0	4.56	1.82	---	1.73	---	1.93	2.70	1.28	1.40	2.10	1.25	1.04	
C15 : 0A	---	---	---	---	---	---	---	---	0.41	---	---	0.62	
C15 : 0	0.53	0.34	---	---	---	---	0.28	0.37	0.21	---	---	0.64	
C16 : 0	29.46	24.20	---	17.61	---	15.98	15.08	14.55	15.63	14.87	16.47	16.97	
C17 : 0A	---	---	---	---	---	---	---	---	0.75	---	---	0.49	
C17 : 0 CYCL	0.18	---	---	---	---	---	---	1.20	---	0.44	---	1.02	
C17 : 0	0.59	0.93	---	0.90	---	1.02	1.05	1.55	1.24	2.08	2.15	1.35	
C18 : 0	7.26	10.53	---	9.10	---	11.18	10.96	11.03	12.52	11.33	11.26	10.52	
C19 : 0	0.11	---	---	0.20	---	---	0.15	---	---	---	---	---	
C20 : 0	---	1.37	---	1.33	---	1.92	1.22	0.94	0.17	0.97	0.61	0.35	
C22 : 0	---	1.03	---	1.14	---	0.52	1.33	1.17	1.26	---	---	0.84	
C24 : 0	0.25	---	---	0.54	---	0.24	0.15	0.30	---	---	---	0.72	
TOTAL SATURATED %	43.39	41.01	---	31.77	---	32.90	33.00	32.45	32.69	31.89	31.74	35.63	
C14 : 1	---	---	---	---	---	---	0.12	---	---	---	---	---	
C16 : 1 W7+W9	6.60	4.70	---	4.93	---	4.49	4.28	4.77	3.91	2.94	2.65	2.51	
C18 : 1 W9	21.85	18.13	---	26.77	---	22.25	25.24	23.81	27.00	28.62	28.96	26.09	
C18 : 1 W7	5.93	7.69	---	9.17	---	9.68	6.78	8.54	6.78	7.13	9.98	8.37	
C20 : 1	1.71	1.41	---	0.99	---	1.28	1.73	1.22	1.00	1.90	1.13	1.14	
C22 : 1 W11	1.07	0.61	---	0.98	---	0.46	0.28	0.55	0.26	0.11	0.43	0.41	
C22 : 1 W9	---	---	---	---	---	---	---	---	---	---	---	---	
C22 : 1 W7	---	---	---	---	---	---	---	---	---	---	---	---	
C24 : 1	---	---	---	---	---	---	---	---	---	---	---	---	
TOTAL MONO-UNSATURATED.	37.16	32.75	---	42.84	---	38.16	38.43	38.98	38.95	40.69	43.15	38.52	
C16 : 4 W1	0.16	---	---	0.34	---	0.49	0.41	---	0.55	---	---	0.58	
C16 : 3	---	---	---	---	---	---	0.09	---	---	---	---	---	
C16 : 2	---	---	---	---	---	---	---	---	---	---	---	---	
C18 : 4 W3	0.50	0.53	---	0.44	---	0.70	0.50	0.39	---	---	---	---	
C18 : 3 W6	0.63	---	---	---	---	---	---	---	---	---	---	---	
C18 : 2 W6	5.05	5.43	---	3.71	---	2.18	3.50	2.79	3.31	4.77	2.66	2.94	
C20 : 4 W6	1.62	4.08	---	2.88	---	3.31	3.43	1.34	3.54	2.26	2.32	2.39	
C20 : 5 W3	5.73	11.57	---	9.15	---	10.49	9.40	13.47	10.83	9.82	9.66	10.61	
C20 : 4 W3	0.15	---	---	---	---	---	0.25	---	---	---	---	---	
C21 : 5	---	0.25	---	0.34	---	0.52	0.39	0.58	---	0.33	0.13	0.25	
C22 : 6 W3	4.62	3.83	---	5.71	---	9.59	9.13	9.50	10.76	9.63	9.09	9.12	
C22 : 5 W3	0.64	---	---	0.54	---	0.59	0.66	0.55	---	0.40	0.28	0.32	
TOTAL POLY-UNSATURATED %	19.40	24.49	---	22.11	---	27.87	27.76	28.50	28.99	27.21	24.14	28.41	

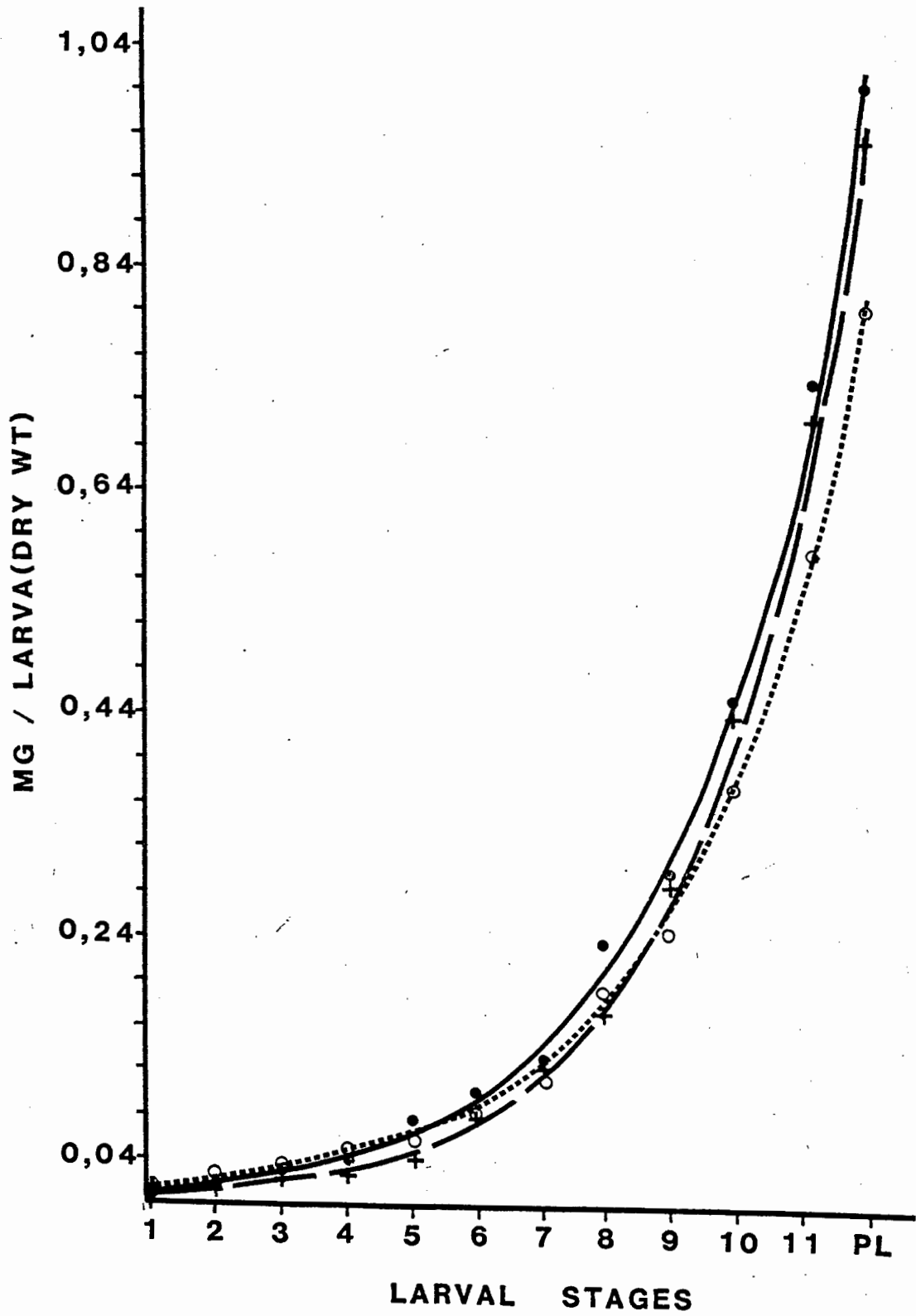


Figure 5: Growth of larvae in cultures 1, 2 and 3.

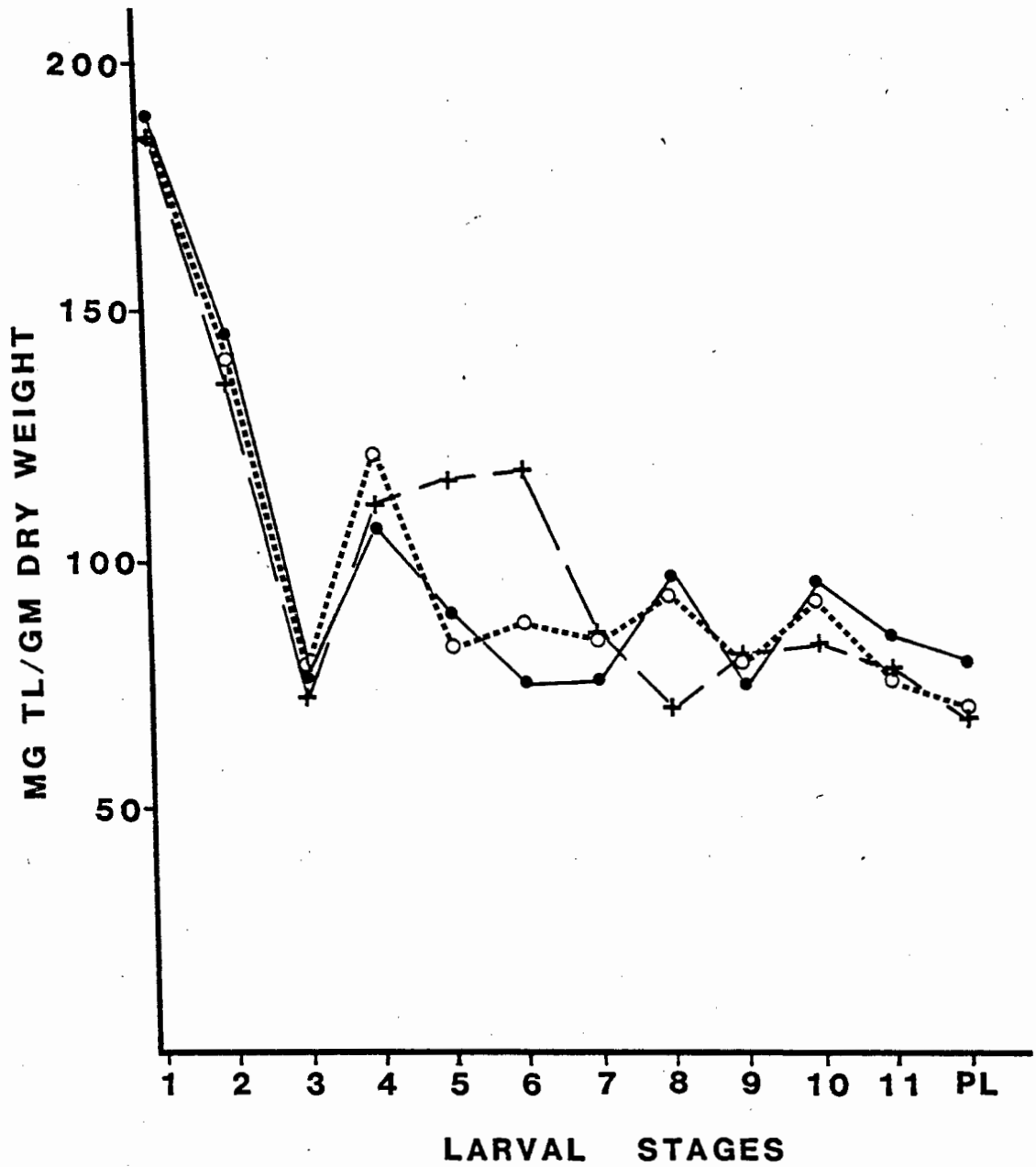


Figure 6: Changes in the total lipid content of the body tissue during the larval development in cultures 1, 2 and 3.

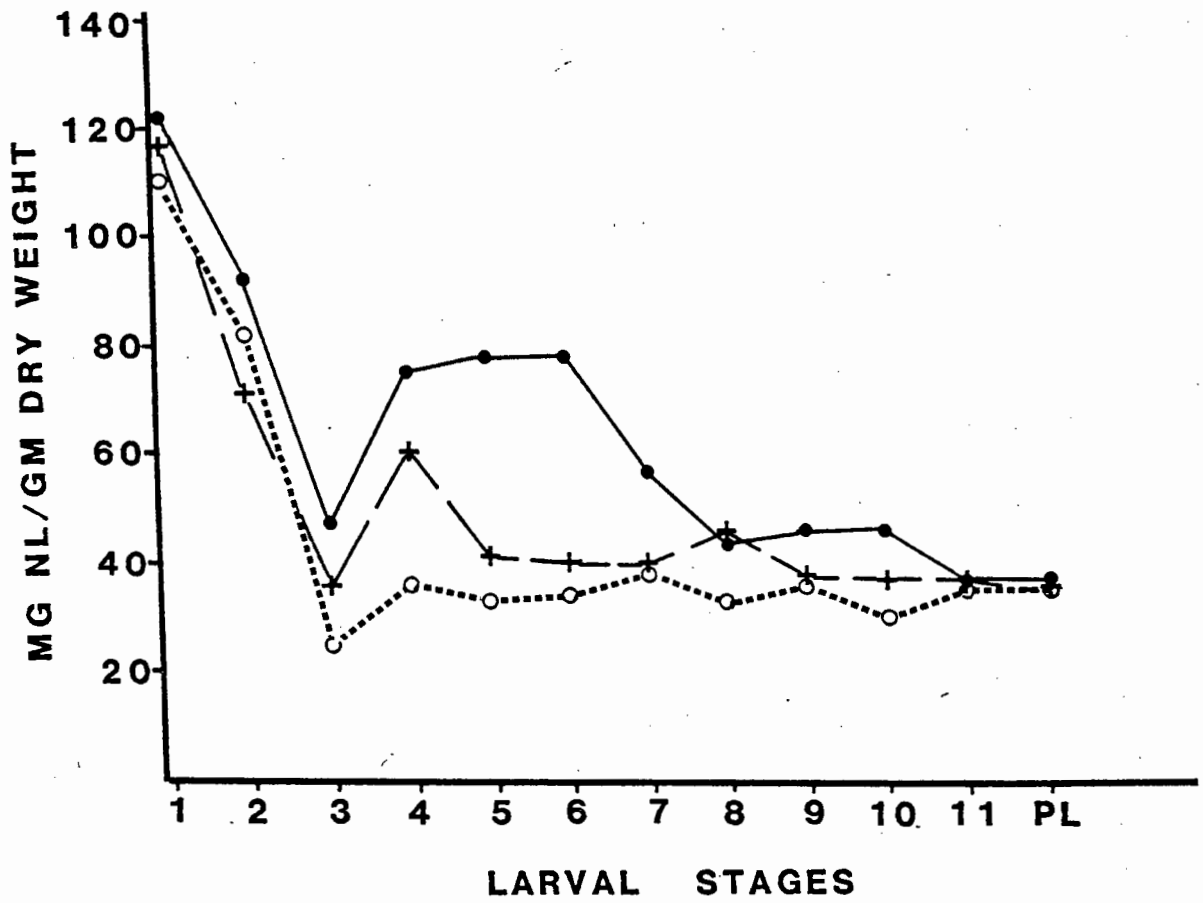


Figure 7: Changes in the neutral lipid content of the body tissue during the larval development in cultures 1, 2 and 3.

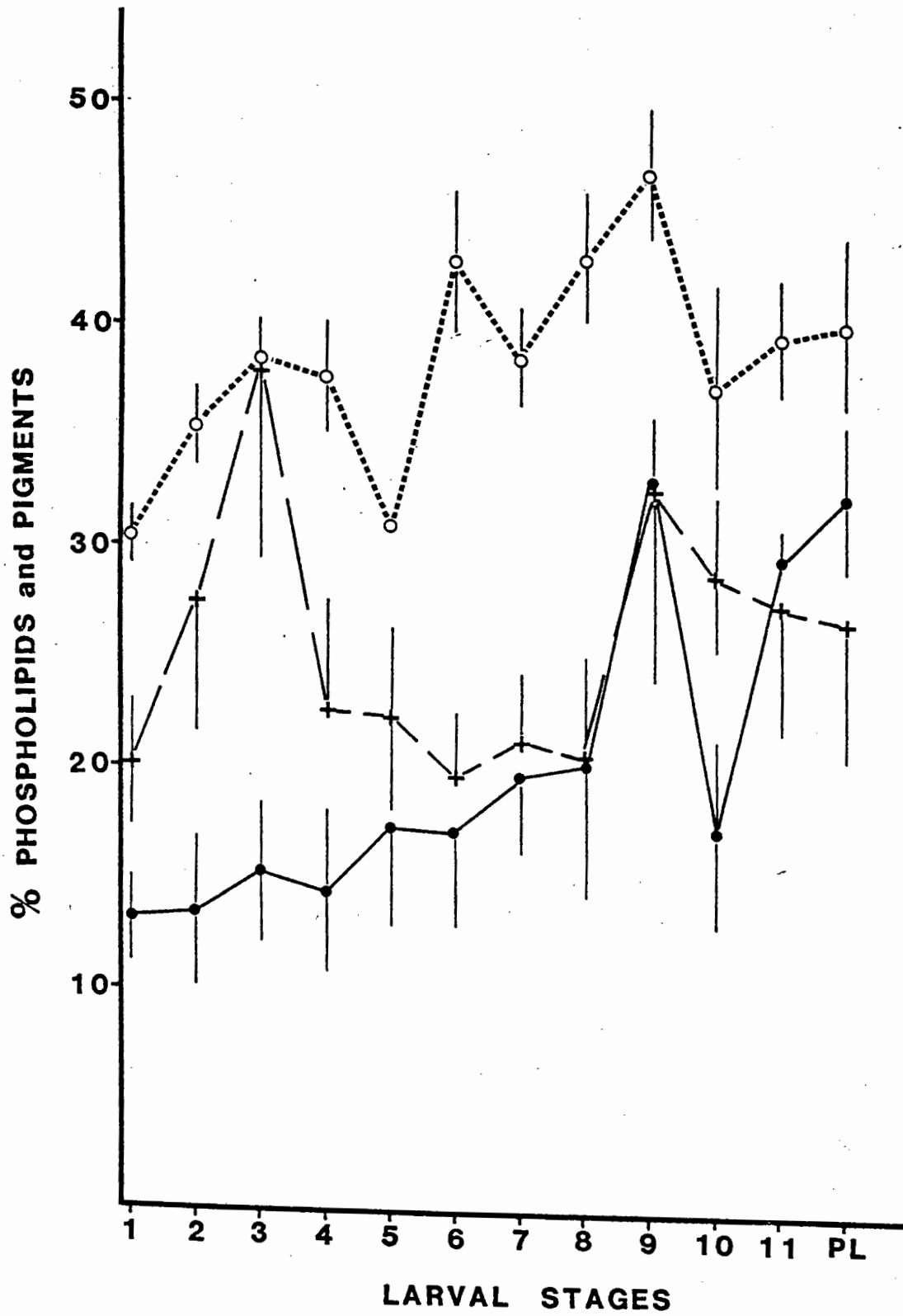


Figure 8: Changes in the percentage composition of the phospholipids and pigments in the total lipid extract of each larval stage during the larval cycles of cultures 1, 2 and 3.

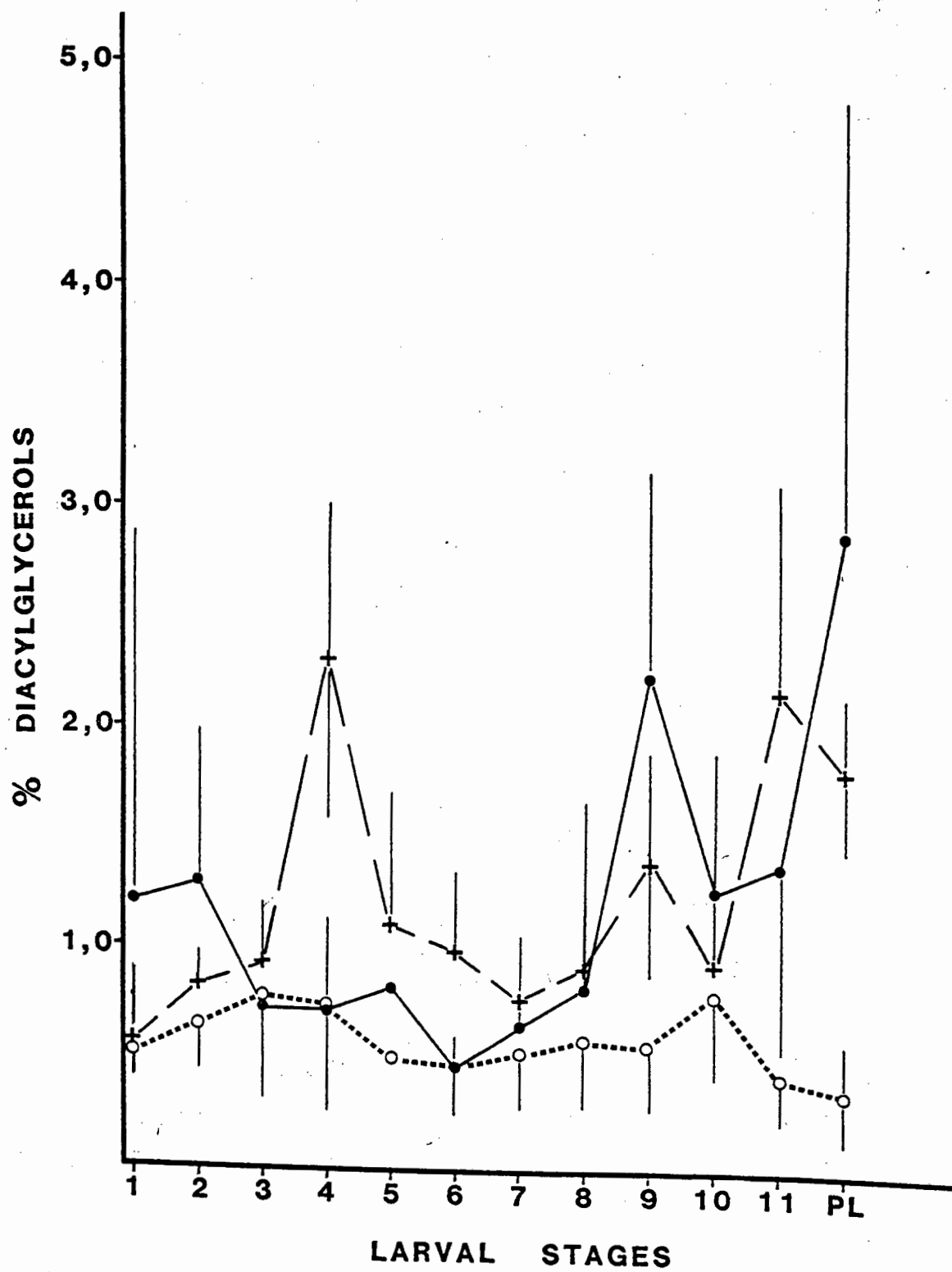


Figure 9: Changes in the percentage composition of the diacylglycerides in the total lipid extract of each larval stage during the larval cycles of Cultures 1, 2 and 3.

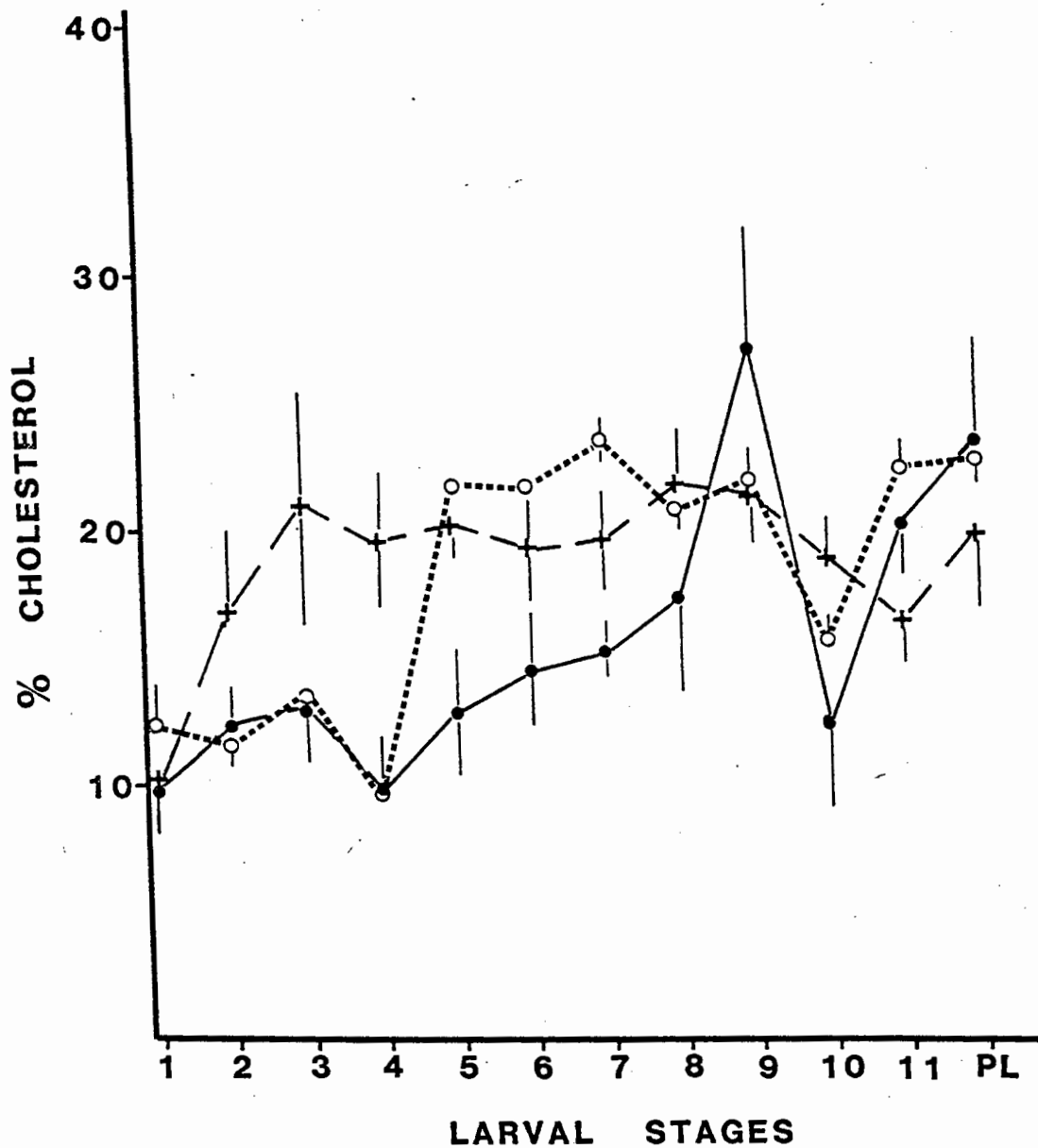


Figure 10: Changes in the percentage composition of the cholesterol in the total lipid extract of each larval stage during the larval cycle of cultures 1, 2 and 3.

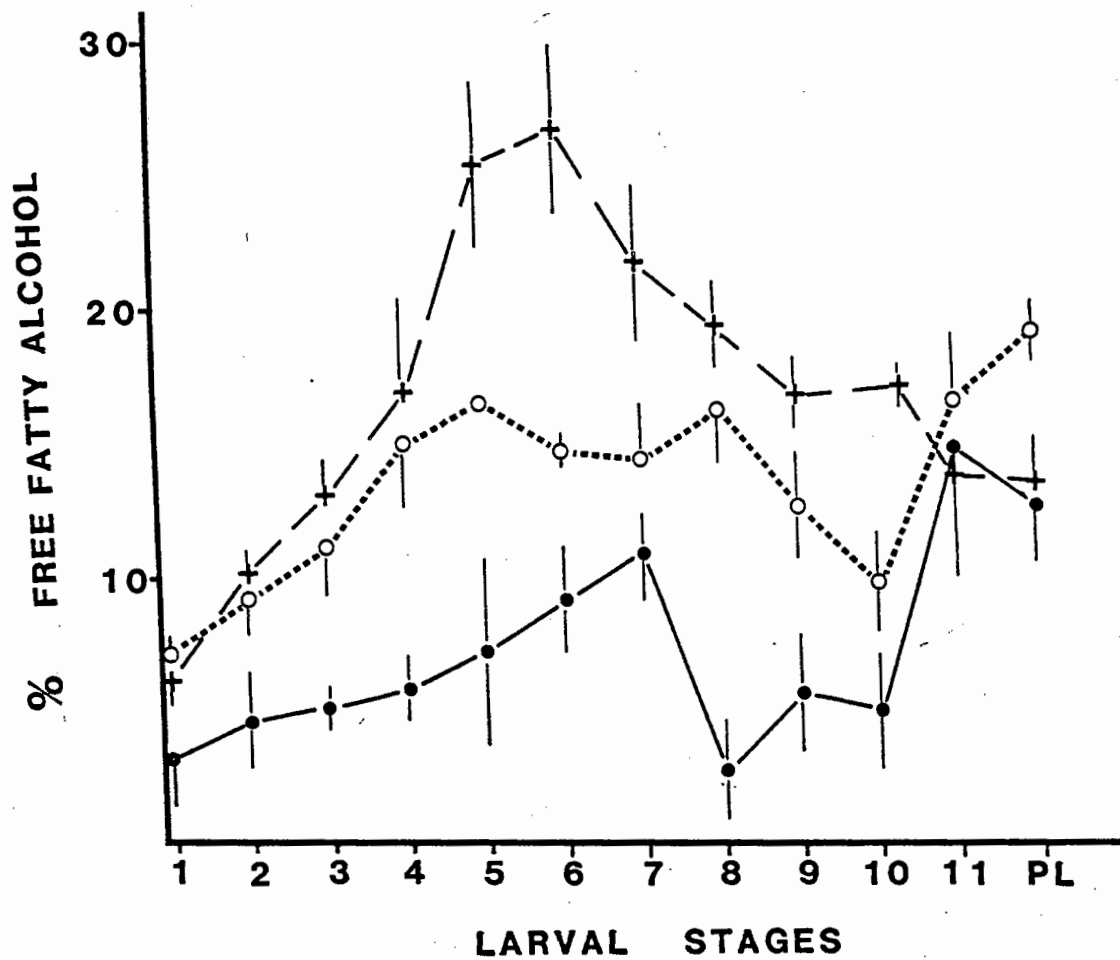


Figure 11: Changes in the percentage composition of the free fatty alcohols in the total lipid extract of each larval stage during the larval cycles of cultures 1, 2 and 3.

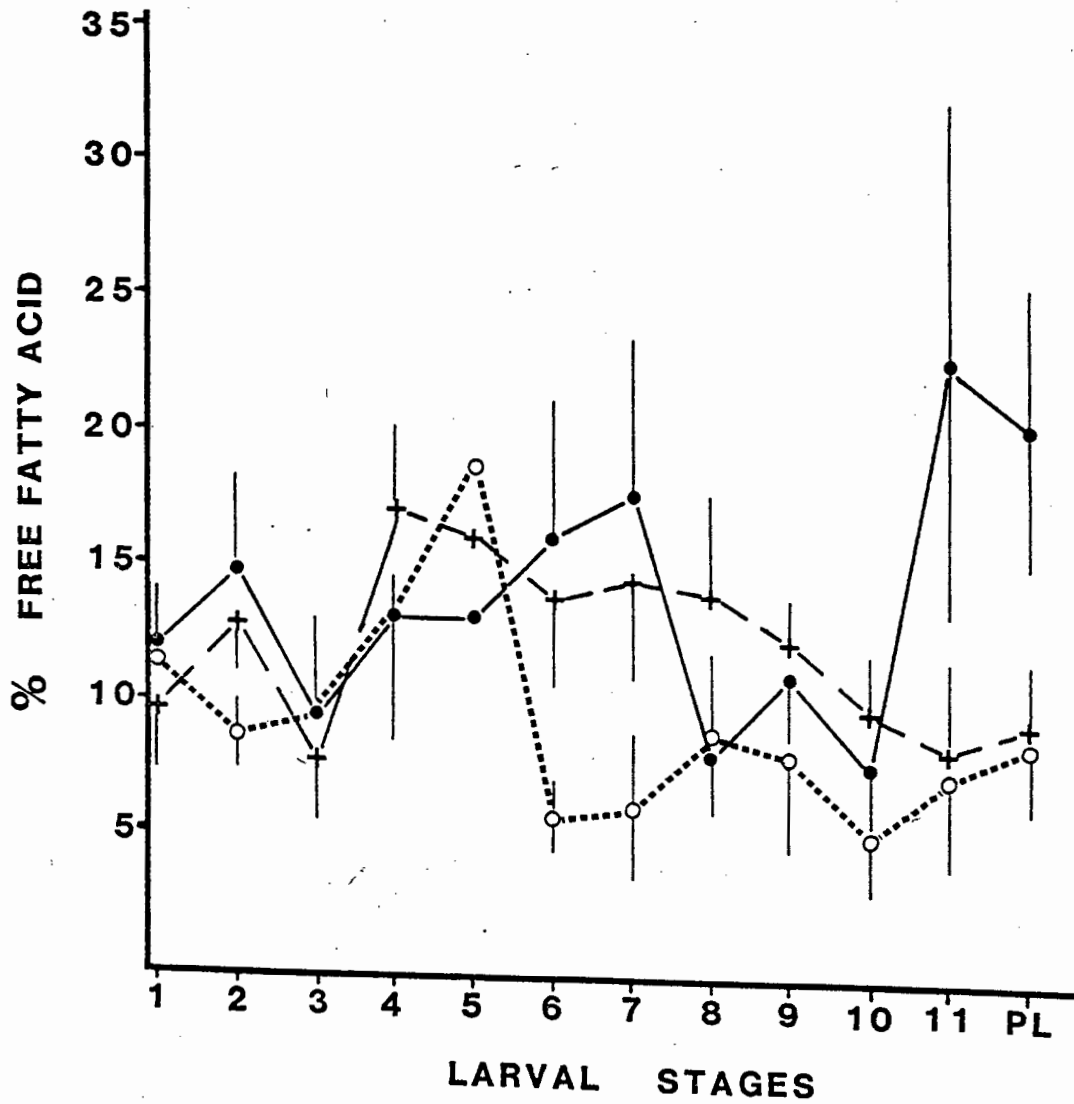


Figure 12: Changes in the percentage composition of the free fatty acids in the total lipid extract of each larval stage during the larval cycles of cultures 1, 2 and 3.

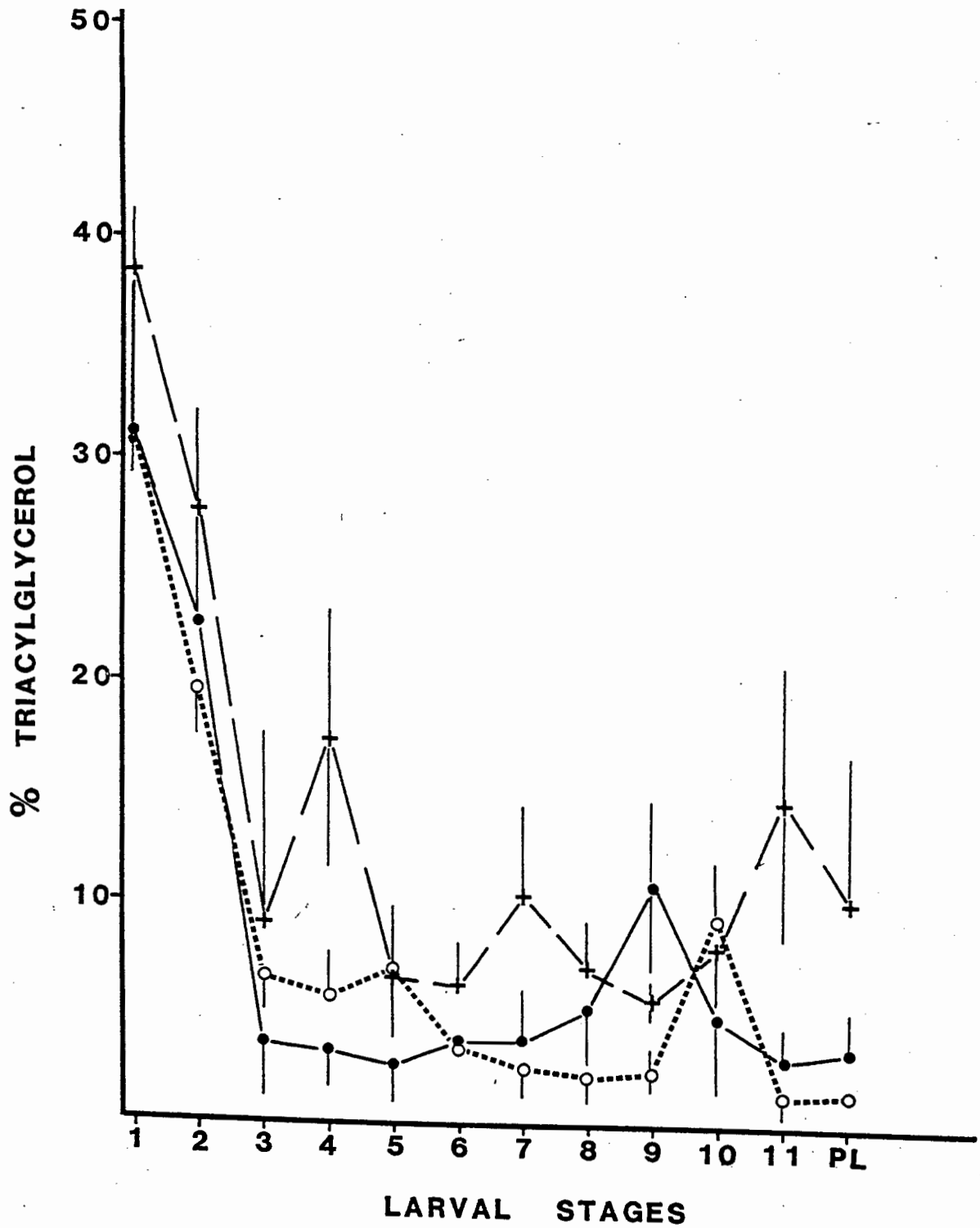


Figure 13: Changes in the percentage composition of the triacylglycerides in the total lipid extract of each larval stage during the larval cycle of cultures 1, 2 and 3.

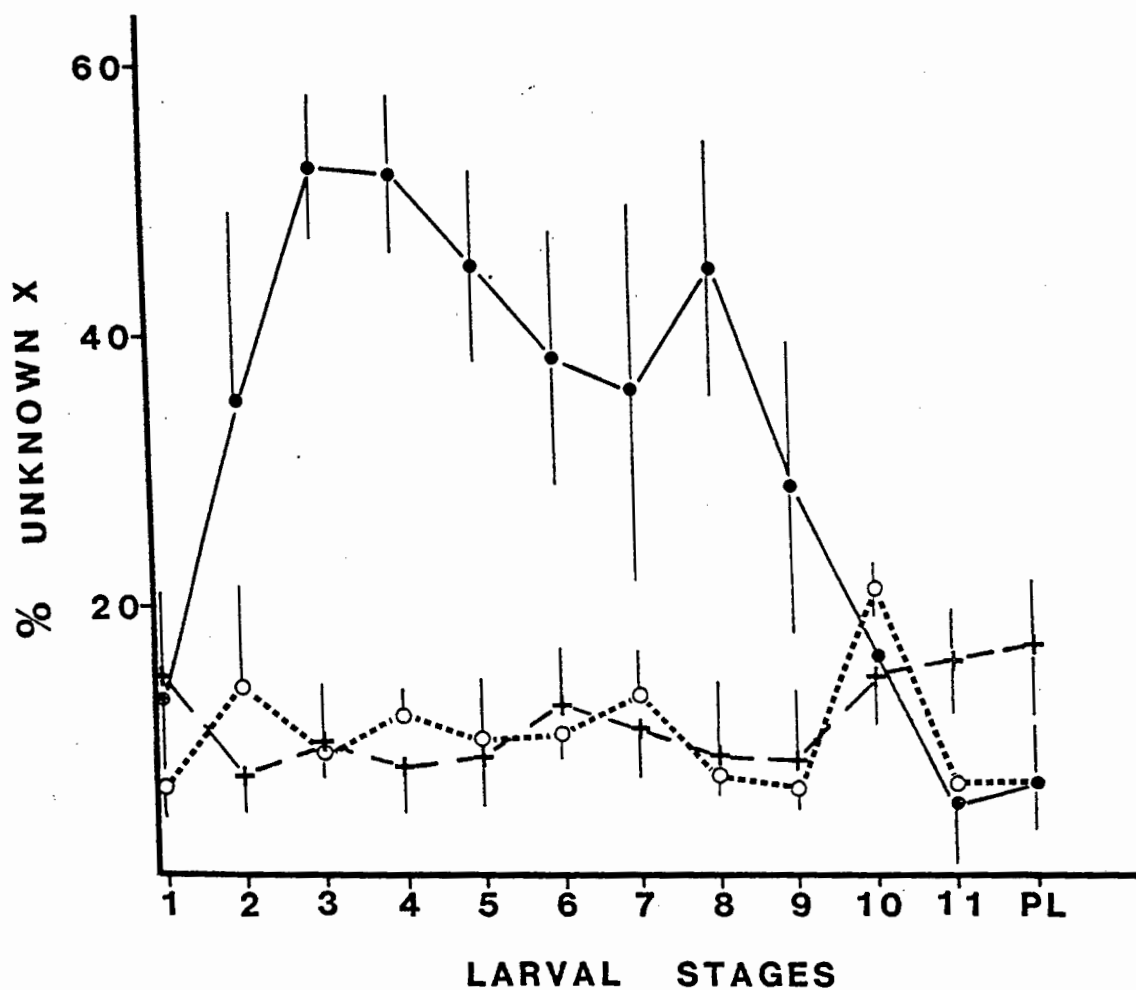


Figure 14: Changes in the percentage composition of the unknown X in the total lipid extract of each larval stage during the larval cycles of cultures 1, 2 and 3.

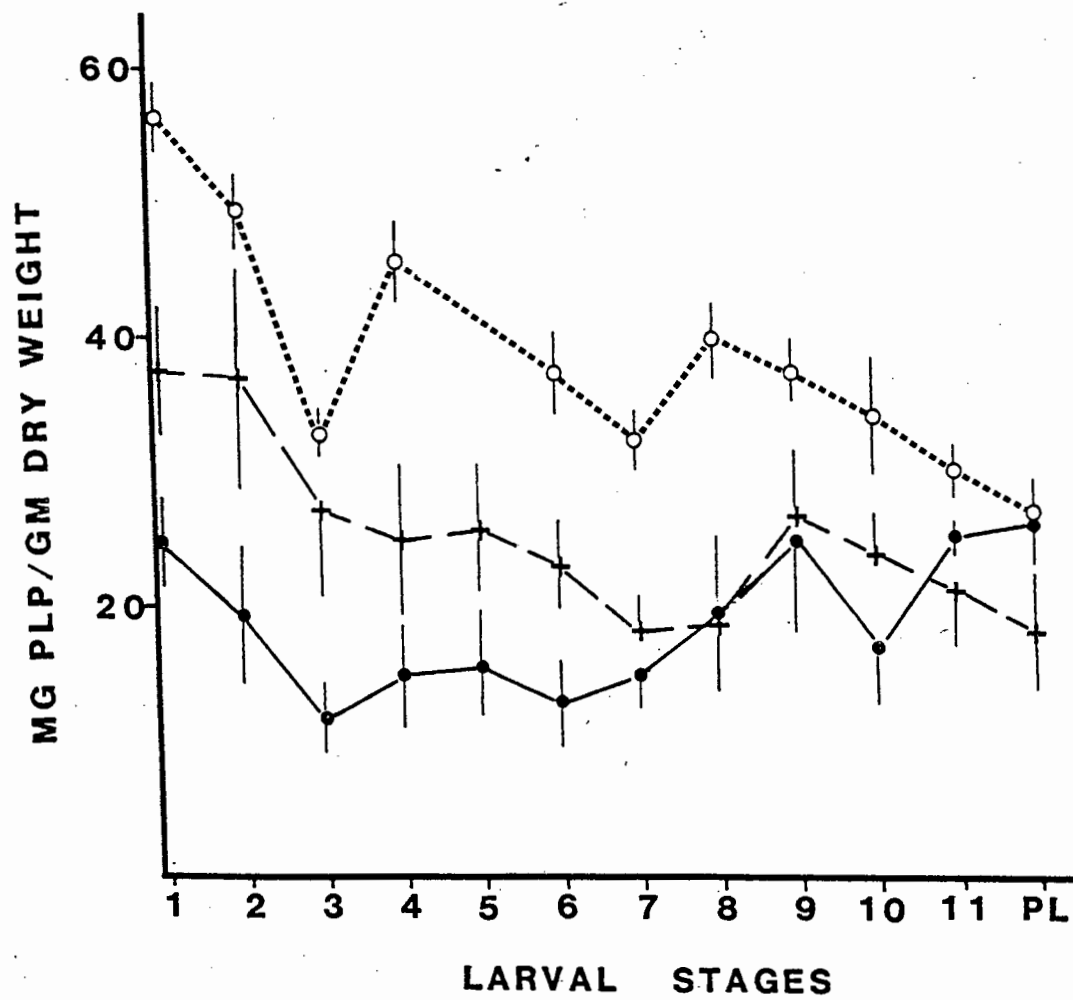


Figure 15: Changes in the body tissue content of the phospholipids and pigments (PLP) of each larval stage during the larval cycles of cultures 1, 2 and 3.

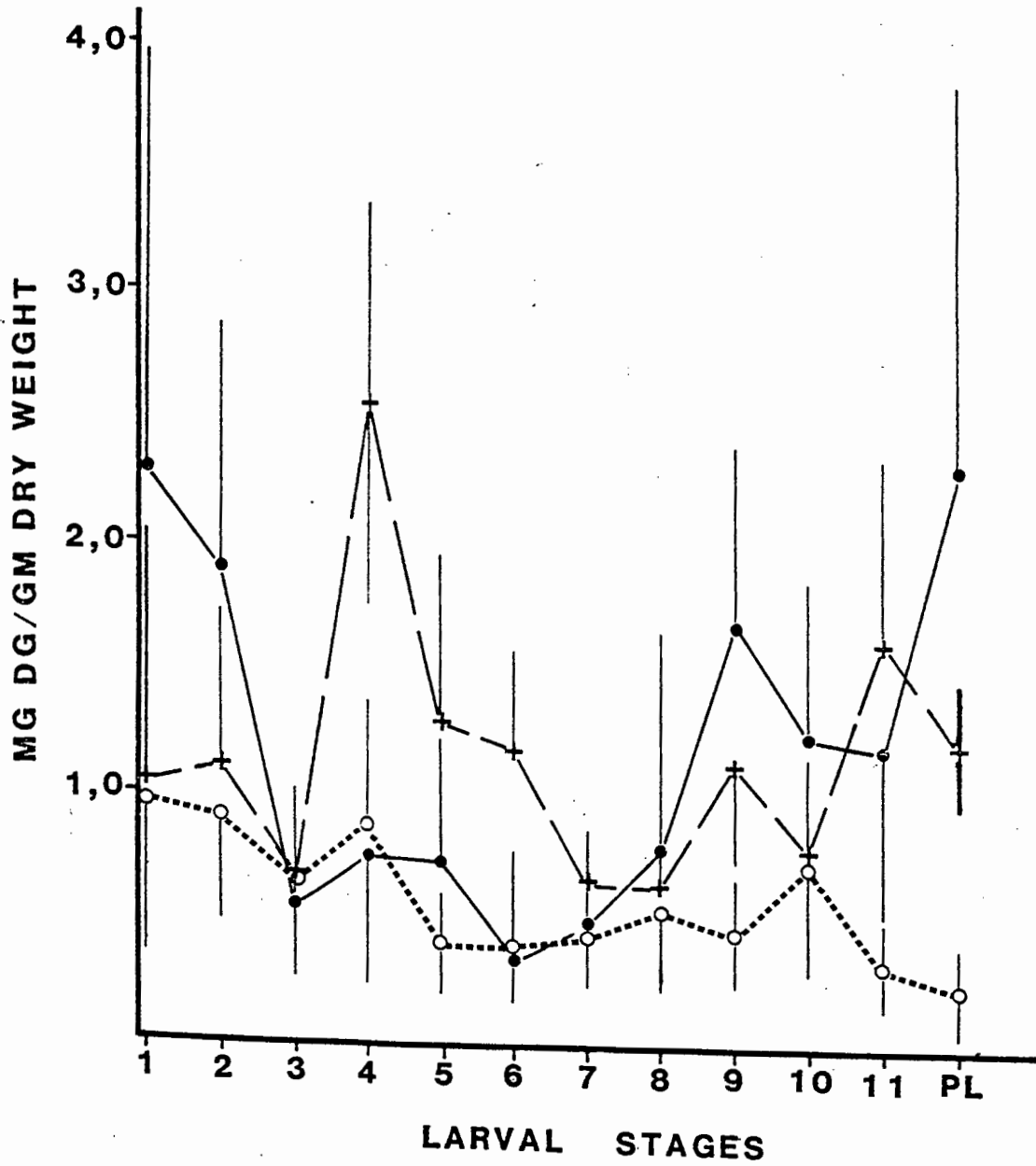


Figure 16: Changes in the body tissue content of the diacylglyceride(DG) of each larval stage during the larval cycles of cultures 1, 2 and 3.

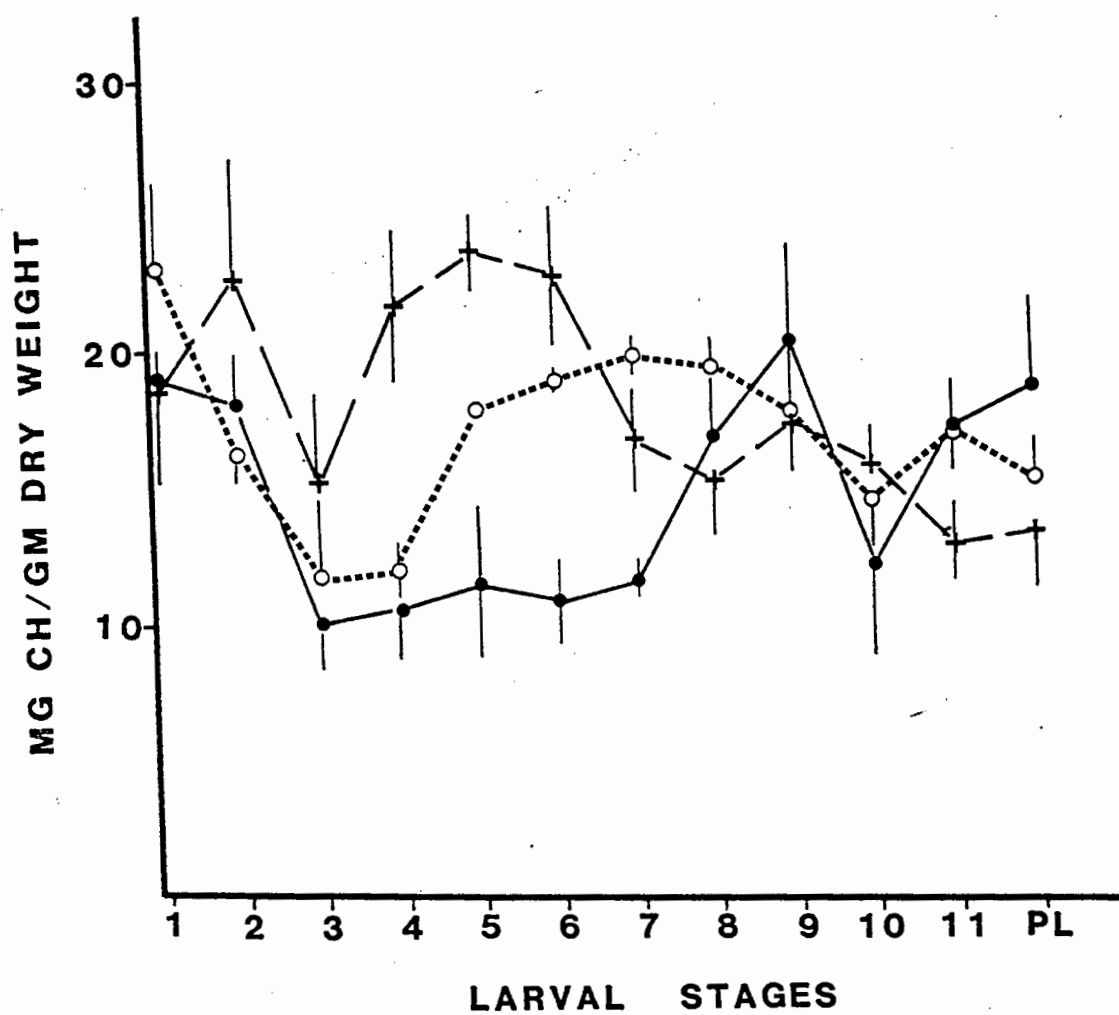


Figure 17: Changes in the body tissue content of the cholesterol (CH) of each larval stage during the larval cycles of cultures 1, 2 and 3.

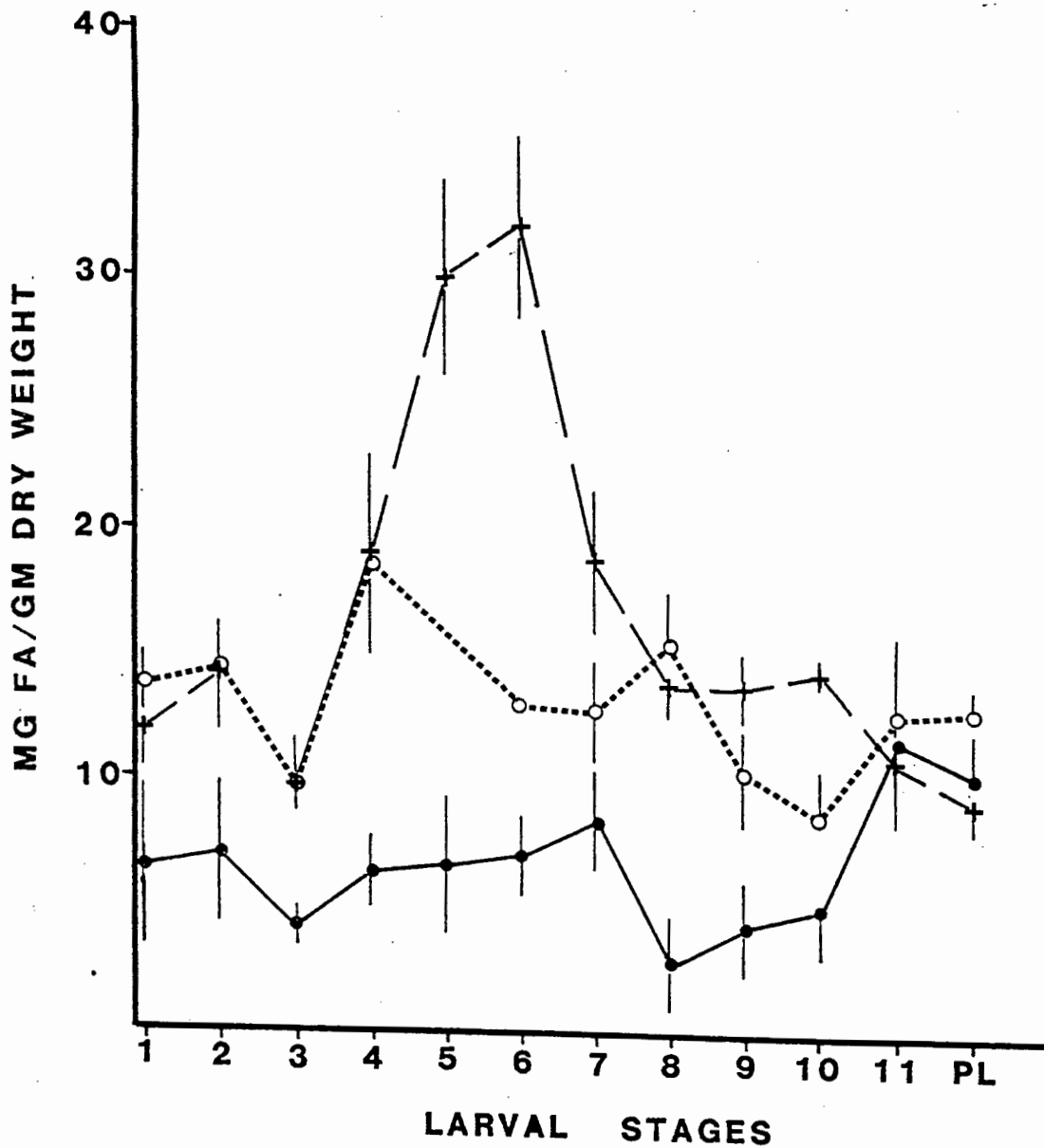


Figure 18: Changes in the body tissue content of the free fatty alcohols (FA) of each larval stage during the larval cycles of cultures 1, 2 and 3.

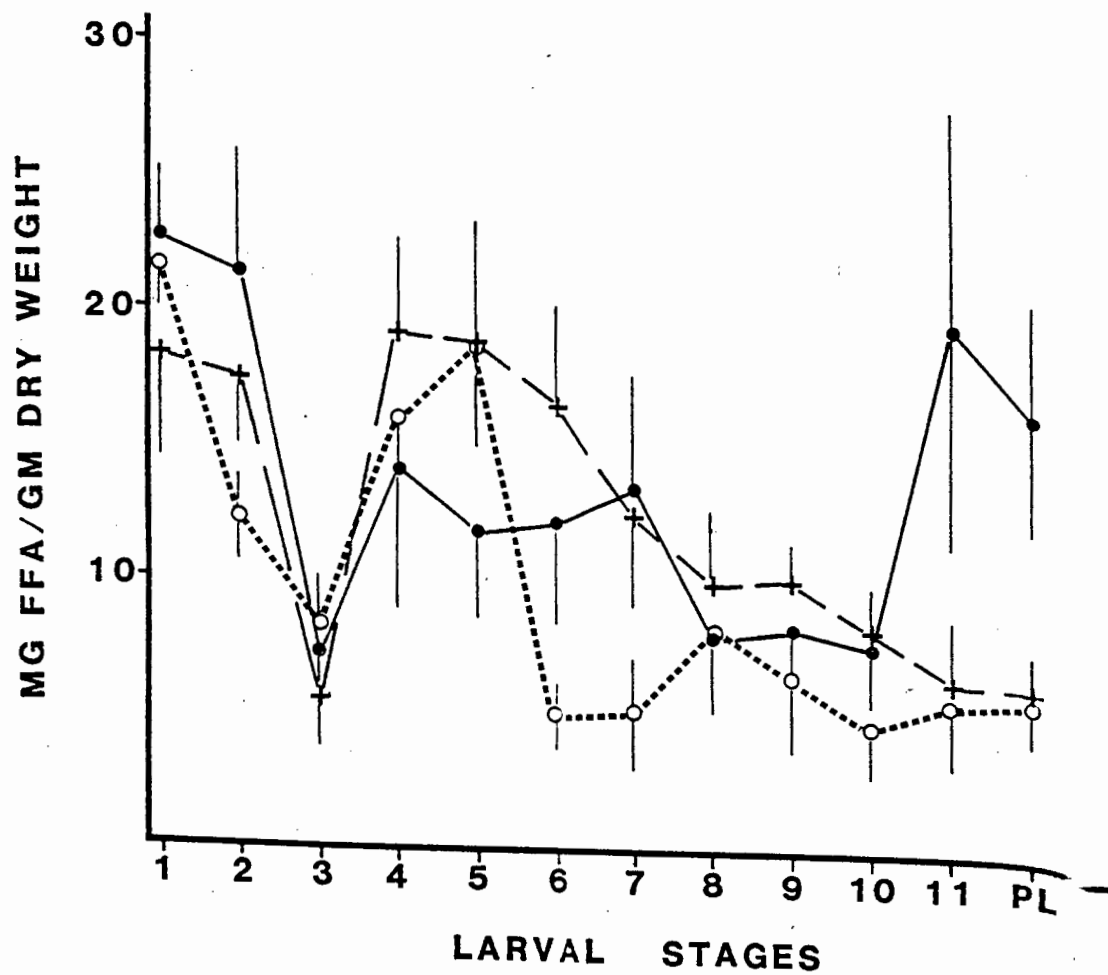


Figure 19: Changes in the body tissue content of the free fatty acids(FFA) of each larval stage during the larval cycles of cultures 1, 2 and 3.

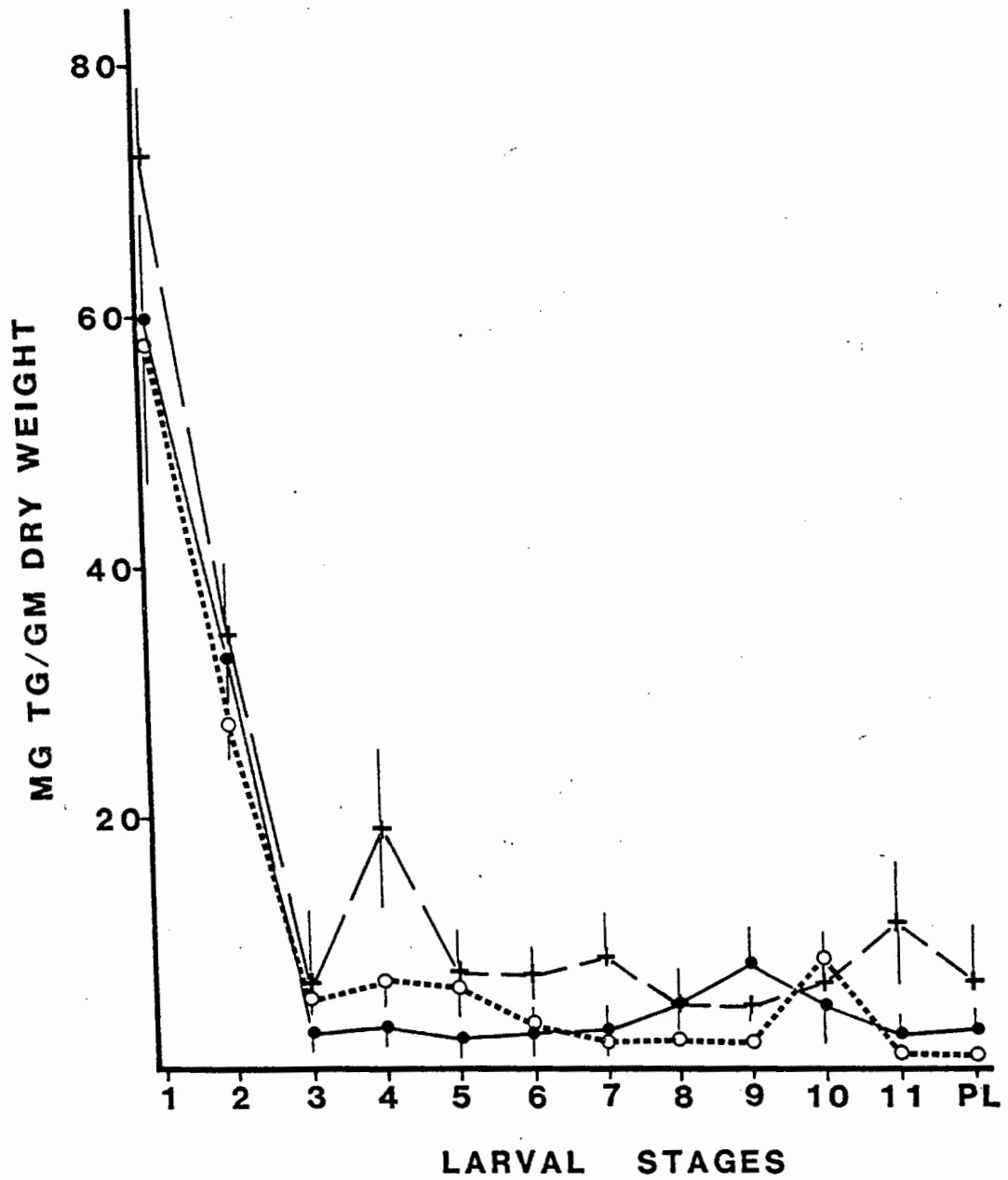


Figure 20: Changes in the tissue content of the triacylglycerides(TG) of each larval stage during the larval cycles of cultures 1, 2 and 3.

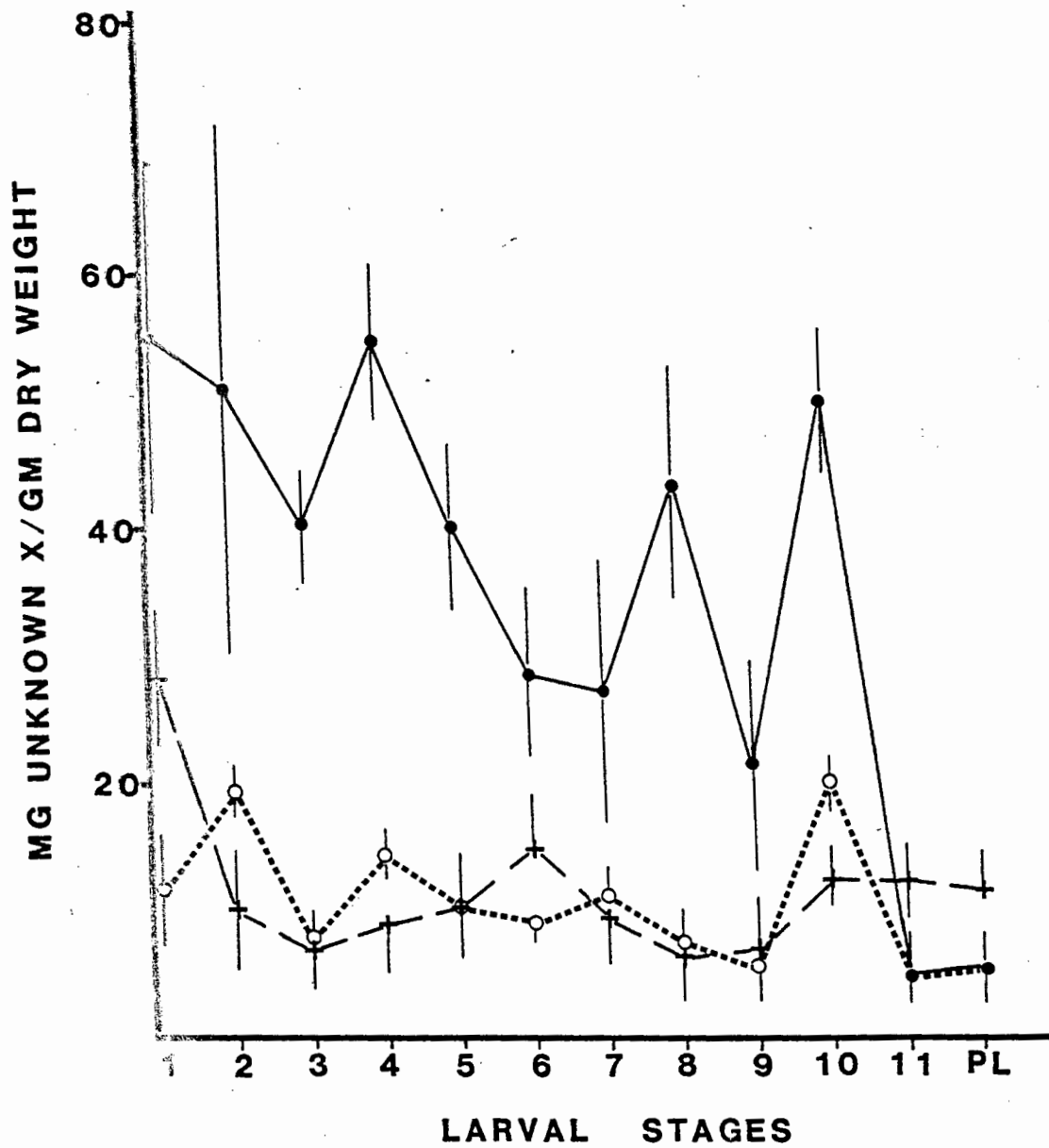


Figure 21: Changes in the body tissue content of the unknown X of each larval stage during the larval cycles of cultures 1, 2 and 3.

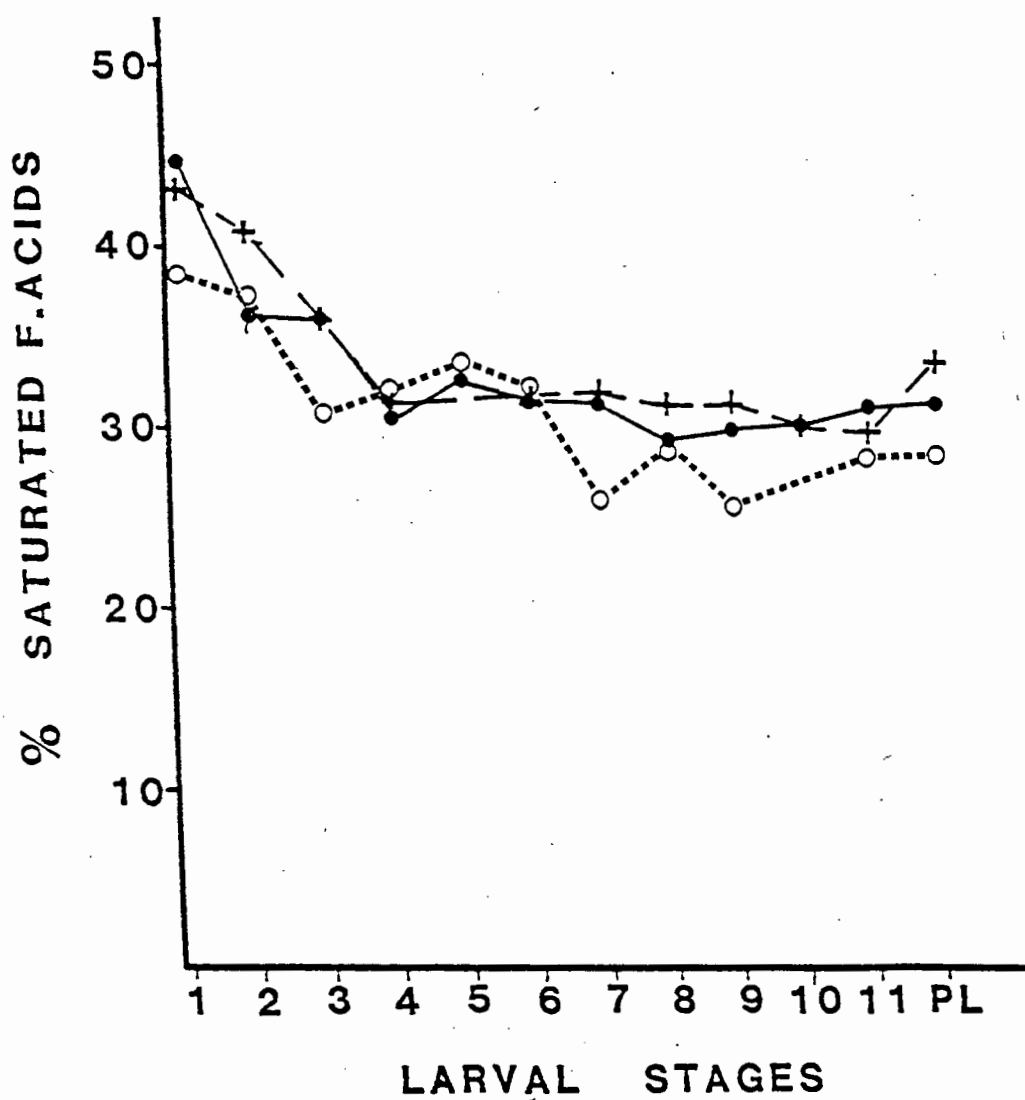


Figure 22: Changes in the percentage composition of the saturated fatty acids in the total methylated sample of each larval stage in cultures 1, 2 and 3.

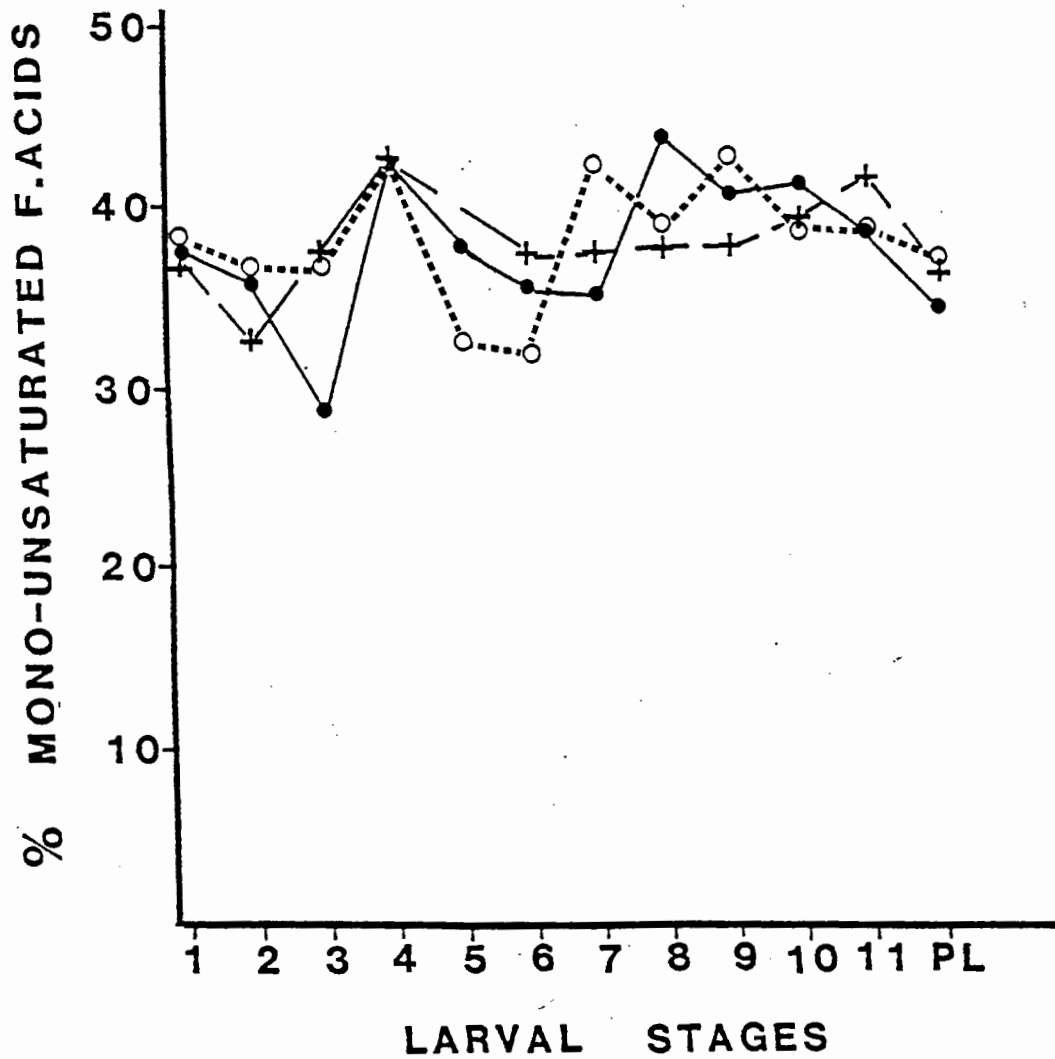


Figure 23: Changes in the percentage composition of the mono-unsaturated fatty acids in the total methylated sample of each larval stage in cultures 1, 2 and 3.

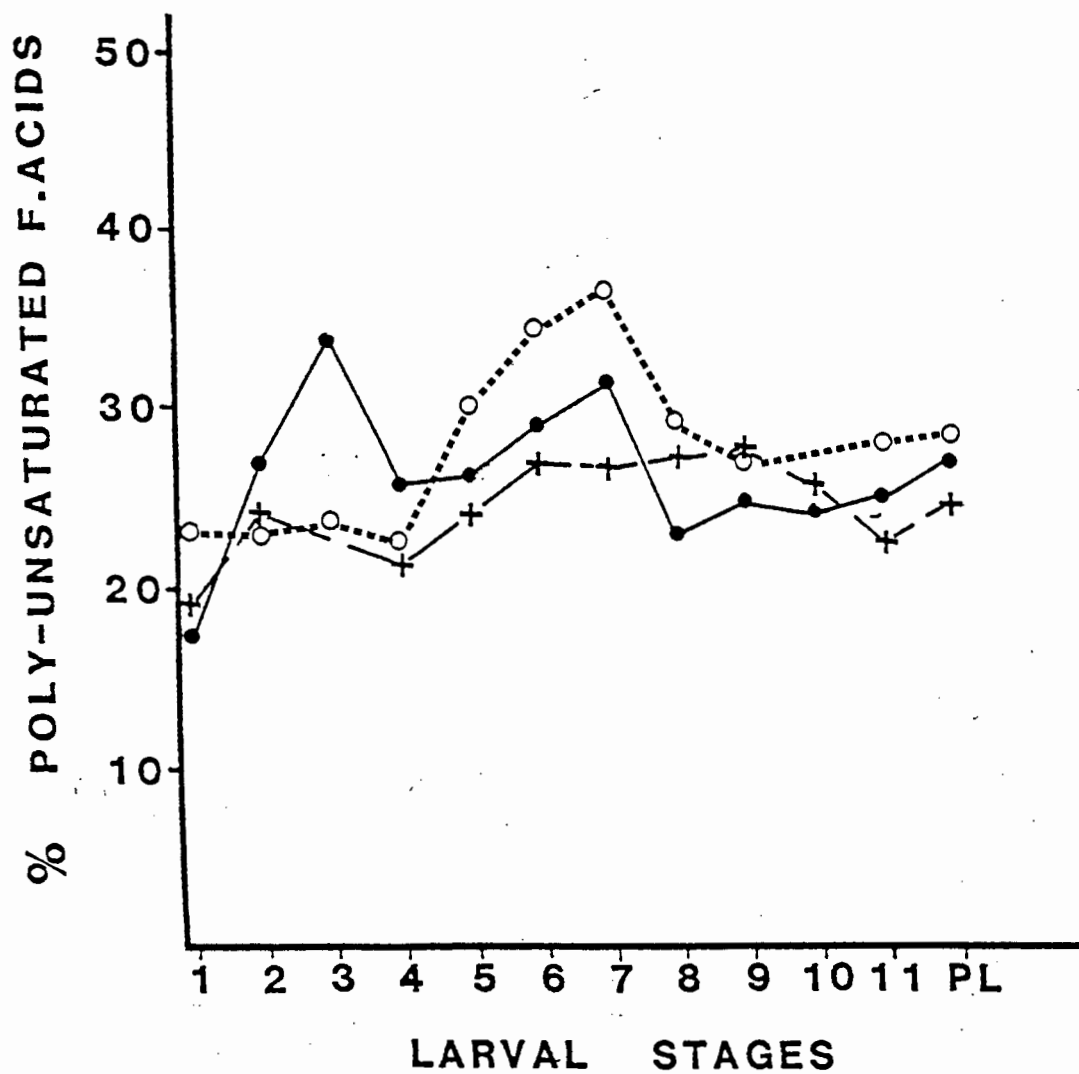


Figure 24: Changes in the percentage composition of poly-unsaturated fatty acids in the total methylated sample of each larval stage in cultures 1, 2 and 3.

PART II

The uptake of dissolved organic compounds by the larvae of  
M. rosenbergii

## INTRODUCTION

Maddox and Manzi (1976), Manzi and Maddox (1976) and Manzi et al (1977) have shown that the presence of algae in the rearing tanks significantly increased growth and survival of the larvae of Macrobrachium rosenbergii, compared to those reared in the absence of phytoplankton. Sandifer et al (1977) reviewed the possible mechanisms by which algae might be beneficial to the larvae. In common with Maddox and Manzi (1976), a brief mention was made of the possibility that algae release metabolites which act as growth factors. The matter, however, was never investigated further.

The last 15 years have seen a renewed and active interest in the uptake of Dissolved Organic Carbon (DOC) by marine animals. Much of the work has been done on adult marine invertebrates (reviews by Jørgensen, 1976; Sepers, 1977; Stewart, 1979). Very little is known about the uptake of DOC by pelagic organisms and crustaceans have largely been neglected.

Work done on DOC uptake in marine larvae is very limited. Manahan carried out a detailed study on the DOC uptake by bivalve larvae (Manahan, 1983; Manahan and Crisp, 1982; Manahan et al, 1982), and most other studies have been restricted to sea-urchin larvae (See review by Pavillon and Vu Tan Tue, 1981). The major emphasis to date has been in simply monitoring the ability of various larvae to take up DOC by adding  $^{14}\text{C}$  and  $^3\text{H}$  labelled soluble organic compounds to the water in the presence of larvae. Thus, very little is known on the

nutritional and ecological importance of DOC in the development and survival of larvae.

From the nutritional point of view, two trophic phases are generally distinguished in the course of larval development: (a) an endotrophic or lecithotrophic phase during which the digestive tract is not yet functional; and (b) an exotrophic phase when the digestive tract is fully developed and functional. During the endotrophic phase, the larvae are said to utilise only their yolk reserves to fulfill their metabolic requirements. The first larval stage (LS1) of M. rosenbergii is lecithotrophic. It is non-feeding and has a large yolk reserve. We have shown in Part IA that the yolk provides the bulk of the energy requirements of LS1 until it moults to the feeding LS2 stage. It is possible that DOC provides some energy to the non-feeding larvae.

Exotrophic larval stages rely essentially on particulate organic matter as a source of food and energy. Manahan (1983) and Pavillon and Vu Tan Tue (1981) show, however, that the uptake and metabolism of DOC by exotrophic larvae can be significant.

Stratham (1975) estimates that DOC in the form of dissolved amino acids absorbed by the egg of the sea urchin Paracentrotus lividus represents about 40% of its energy requirement and that absorbed by the 48 hour pluteus, 10% of its energy requirements. It is possible, however, that DOC may have a greater nutritional importance for exotrophic larvae at times of shortages of particulate food.

Dissolved organic matter constitutes the predominant part of the total organic matter present in natural waters. According to Jørgensen (1976), the amount of organic matter dissolved in the oceans equals the total of more than 30 years of primary production. A good account of the origin of DOC is given by Pavillon (1981). In the oceans the DOC is in the form of small molecules such as amino acids, glucose and other carbohydrates, fatty acids and vitamins. These are produced mainly in the euphotic zones to which they are restricted. High concentrations of DOC may, however, be produced by microbial activity in organic rich sediments and remain locked in the interstitial water and to the zone just above the sediments (Barber, 1968).

The situation in estuaries, where the larval stages of M. rosenbergii occur, is quite different. Along with sediments carried down by rivers, a large amount of organic matter in particulate and dissolved form is added to the system by both the rivers and the sea. In addition, there is organic matter resulting from the excretion and decomposition of estuarine animals and plants. Plant exudates and animal excretions contribute most of the DOC whereas the detritus produced from the death of organisms is primarily in the form of particulate matter (McLusky, 1981). Table 8 shows the concentrations of organic carbon in natural waters, and this indicates that the level of DOC in estuaries is much higher than in the sea.

TABLE 8: Concentrations of organic carbon in natural waters. Figures in brackets represent extreme values (After McLusky, 1981).

Concentration (mg L <sup>-1</sup> ) of Organic Carbon	River	Estuary	Coastal Sea	Open Sea		Sewage
				Surface	Deep	
Dissolved (DOC)	10-20(50)	1-5(20)	1-5(20)	1-1,5	0,5-0,8	100
Particulate (POC)	5-10	0,5-5,0	0,1-1,0	0,01-1,0	0,003-0,1	200
TOTAL	15-30(60)	1-10(25)	1-6(21)	1-2,5	0,5-0,8	300

In deep, slow-flowing estuaries which often occur in Southeast Asia where M. rosenbergii larvae are found, the long residence time of estuarine water allows several generations of planktonic organisms to develop during a single year. Phytoplankton blooms often occur (McLusky, 1981) and these will produce a considerable amount of DOC.

Algal excretion of organic compounds is well documented (Fogg, 1962; Le Fevre, 1964; Nalewajko, 1966). Hellebust (1965) determined the excretion of photoassimilated carbon for 22 species of marine algae. He found that most algae excreted 3 - 6% of their photoassimilated carbon during periods of logarithmic growth. A few species excreted as much as 10 - 25% under the same conditions. The excretion of organic carbon was very high, 8,5 - 52% of the photoassimilated carbon, when incubated outdoors for five hours in full sunlight. In fresh water algae, losses of up to 50% have been reported (Fogg et al, 1965; Watt, 1966). The marsh grass, Spartina alterniflora, releases DOC into the Patuxent river estuary in North America at the rate of 61 kg C ha<sup>-1</sup> yr<sup>-1</sup> (Gallagher et al, 1976).

Wieber and Smith (1977) found that algae have higher exudation rates when incubated in light rather than in darkness. They also found evidence that the exudates do not arise from cell damage or lysis during incubation. Hellebust (1965) and Belly et al (1973) report that most of the substances excreted by algae are of low molecular weight, and include such compounds as amino acids, polysaccharides, peptides, vitamins, steroids and saturated and unsaturated fatty acids. Different algae, though, may excrete different products. Dunaliella tertiolecta, for example, excrete mainly the sugar-alcohol glycerol, whilst the marine Chlorella sp. excrete mostly the amino acid proline. Bacteria, like algae, are producers of vitamins, amino acids, polypeptides and polysaccharides (Pavillon, 1981).

Thus, in tropical and equatorial estuaries, a wide spectrum of low molecular weight dissolved organic products may be found in fairly high concentrations. For M. rosenbergii larvae, which grow in this type of environment, DOC may have some significance as a supplementary energy source, especially in times of particulate food shortages.

The possible role played by vitamins dissolved in water on larvae has been investigated to date only by Pavillon and his associates. Pavillon (1976a), studying the uptake of riboflavine, thiamine and cobalamine (vitamins B12, B1 and B2 respectively) in the egg and 48 hour nauplius of Artemia salina, and in the 48 hour larva of the echinoid Arbacia lixula, showed that, in the eggs and larvae of Artemia salina, the absorption of vitamins is greater than that of amino acids. In Arbacia lixula, however, it is the amino acids which

are most absorbed. Pavillon and Wu Tan Tue (1981) have shown uptake of riboflavine and cobalamine by the larvae of the fish Dicentrarchus labrax, though in lower quantities than that of amino acids. They have also investigated the uptake of vitamins in the krill Euphansia krohnii (Pavillon, 1977).

Pavillon (1981) reports that in the extrophic phase of larvae, namely in Artemia and the urchin pluteus, the uptake of DOC fulfills only a small percentage of the total energy requirement. He observes, however, an increase in the uptake of labelled vitamins dissolved in the water by the pluteus, this conforming with the increasing need for these compounds as the larvae grow.

An increase in the growth rate and a shorter time to metamorphosis of larvae in nature is important for survival as it reduces the chance of predation. In commercial mass culture systems this has considerable importance as a shorter larval cycle might produce healthier animals and also allow an increase in the production turnover of post larvae for stocking in grow out ponds.

The aim of the present study was to determine whether M. rosenbergii larvae take up DOC in the form of  $^{14}\text{C}$ -glycine,  $^{14}\text{C}$ -glucose and exudates from phytoplankton. An attempt was also made to determine the fate of the  $^{14}\text{C}$ -glycine absorbed by the larvae and its metabolic significance. In the present study the uptake of vitamins was not investigated.

## MATERIALS AND METHODS

### Animals

The larvae were reared in the facilities described in the section on larval rearing. All investigations were conducted on late stage 2 larvae. For each series of experiments, larvae were counted, freeze-dried and weighed so that DOC uptake could be calculated on a tissue dry weight basis.

Throughout the uptake experiments the temperature was kept at 28°C  $\pm 1^\circ$  C and the salinities at 12 ‰.

### Gut Clearance Time

Larvae were fed with fish and artemia and examined under the microscope at 5-minute time intervals for the first half-hour and at 15-minute time intervals thereafter to determine how long it took for the food to pass through the gut of the larvae.

### Incubation Media

All glassware was sterilised by autoclaving at 120°C for 20 minutes. For all experiments the larvae were placed in sterile-filtered (0,2  $\mu$ M Millipore), autoclaved water (SFAW) at the appropriate temperature and salinity. Before all experiments, the animals were transferred to 3L of SFAW for three hours. This was to allow time for complete gut

clearance. The larvae were then rinsed in fresh SFAW and transferred to 200 ml of SFAW for about 15 minutes for a final rinsing before transfer to the experimental containers.

For single-substrate experiments ( $1\text{-}^{14}\text{C}$ ) glycine (54,2 mCi/mmol) and  $\text{U-}^{14}\text{C}$ ) glucose (270 mCi/mmol) were added to the containers to give the required concentrations. The glycine label was initially diluted so that 100  $\mu\text{l}$  of label contained 1  $\mu\text{Ci}$  of  $^{14}\text{C}$ -glycine. Thus, for experiments conducted at 0,4  $\mu\text{M}$  concentration 2,5  $\mu\text{Ci}$  or 250  $\mu\text{l}$  were added to 100ml SFAW. This can be calculated from the given specific activity of the label (54,2 mCi/mmol). The calculations for the glucose concentrations were made in the same way using the given specific activity of 270 mCi/mmol.

For the algal exudate experiments, 2L of Phaeodactylum tricornutum at a cell concentration of about  $1 \times 10^6$  cells  $\text{ml}^{-1}$  was labelled with 400  $\mu\text{Ci}$   $\text{U-}^{14}\text{C}$  sodium bicarbonate for 48 hours and aerated in fluorescent light. The algae were then centrifuged for 10 minutes at 1500 rpm and the water poured out. The cells were rinsed and centrifuged three times with new SFAW before being finally suspended in one litre of SFAW and allowed to stand for 24 hours in fluorescent light whilst being aerated for exudation to take place. The cells were then centrifuged at 8000 rpm for 15 minutes and the water rich in algal exudates but free of algal cells was then used for the experiments.

The amount and ratios of dissolved free amino acids were determined for the exudates of an unlabelled culture of the same concentration. Determination was done on a Beckman 114 HPLC.

### Time Course Experiments

Twenty-five larvae were placed in each conical flask labelled with  $^{14}\text{C}$ -glycine at 0,4  $\mu\text{M}$  concentration for the glycine series, and with  $^{14}\text{C}$ -glucose at 0,8  $\mu\text{M}$  for the glucose series. Separate flasks of larvae were terminated at times 0, 30 minutes, 1 hour, 3 hours, 6 hours, 15 hours, 24 hours and 46 hours for the glycine experiments and times 0, 1 hour, 2 hours, 4 hours, 12 hours, 18 hours and 24 hours for the glucose series. A parallel time course experiment with  $^{14}\text{C}$ -glycine for  $^{14}\text{CO}_2$  production was performed at times 0, 1 hour, 3 hours, 6 hours, 15 hours and 24 hours.

Though it was not possible to calculate the molarity of the radio label in the algal exudate experiments, the above experiments for tissue uptake at times 1 hour, 3 hours, 6 hours, 13 hours and 24 hours of labelled algal exudate was performed.

All experiments were carried out in triplicate.

### Measurement of $^{14}\text{CO}_2$ Production

To collect the  $^{14}\text{CO}_2$  produced by the larvae, 50 larvae were placed in each flask in 50 ml of SFAW with label at 0,4  $\mu\text{M}$  concentration. In the base of each flask a 5 ml glass vial was fixed. One ml of 10% KOH was pipetted into this vial. The system was sealed by a greased rubber bung through which a bent glass tube had been inserted. To the outside end of the glass tube, a piece of rubber tubing was fixed and

closed with a clamp to seal the unit. A separate flask was used for each determination.

All experiments were carried out in triplicate.

At a given time interval, 3 ml of concentrated HCL was injected through the closed rubber tubing down the bent glass tube into the water containing the larvae. This effectively stopped the experiment and displaced the  $^{14}\text{CO}_2$  from the water by acidification. The flask was allowed to stand for about 18 hours during which time the  $^{14}\text{CO}_2$  was absorbed by the KOH. The flask was then opened, the KOH removed and placed in a scintillation vial containing 1 ml methanol and 10 ml Beckman Ready Solve HP/b. The methanol was added to prevent the formation of a precipitate on addition of the scintillation fluid. Manahan (1983), using a similar technique, recovered 95% of  $^{14}\text{CO}_2$  produced. Blank runs without larvae were conducted as controls.

#### Biochemical Fate of Amino Acid Carbon

Using methods modified from Holland and Gabbott (1971), homogenates of larvae were used to obtain extractions of protein and lipid fractions. As it was difficult to obtain a fine homogenate for uniform subsampling, it was decided that each test should be run separately, in triplicate. Fifty larvae were used for each replicate. Analysis was done for larvae exposed for three hours and 24 hours in  $^{14}\text{C}$ -glycine.

### Lipids

3 x 50 larvae were homogenised with chloroform, methanol and water according to the method of Bligh and Dyer (1951) (Appendix 2). This method was explained in detail in the section on Lipids. After centrifugation at 5000 rpm, the chloroform containing the lipid was removed to a scintillation vial and the supernatant washed with the same volume of chloroform which was added to the scintillation vial after centrifugation. The chloroform was then evaporated to dryness under a stream of nitrogen.

### Protein

3 x 50 larvae were homogenised and taken through the process given in Table 9.

### Total Tissue Incorporation of Label

3 x 50 larvae were sampled at 3 hours and 24 hours and processed for scintillation in the method given for preparation for scintillation count. It was thus possible to subtract from these the protein and lipid values to obtain the TCA soluble fraction (i.e. carbohydrates and small molecular weight compounds). Knowing the amount of  $^{14}\text{CO}_2$  produced for the two times it was thus possible to determine the fate of  $^{14}\text{C}$ -glycine taken up by the larvae.

TABLE 9

Scheme for the determination of the percentage of  $^{14}\text{C}$  in the lipid, protein and TCA soluble fractions of M. rosenbergii larvae.

LIPID	PROTEIN	TOTAL
3 x 50 larvae replicates	3 x 50 larvae replicates	3 x 50 larvae replicates
Add 200 ul $\text{H}_2\text{O}$ 200 ul $\text{CHCl}_3$ 500 ul $\text{CH}_3\text{OH}$ Homogenise and shake for 1 min. Stand at $4^\circ\text{C}$	400 ul $\text{H}_2\text{O}$ Homogenise 200 ul 15% TCA Shake 1 min. Stand at $4^\circ\text{C}$ for 10 min. Centrifuge at 5000 rpm for 5 min. Remove all supernatant. Wash ppt x 3 with 500 ul 5% TCA Centrifuging and removing each time. Ppt $\equiv$ protein.	Add 500 ul Soluene 350 tissue solubilise and digest for 2 hours at $50^\circ\text{C}$ .
Add 200 ul $\text{CHCl}_3$ Shake for 1 min	Add 500 ul Soluene 350 Digest for 2 hours at $50^\circ\text{C}$ .	
Add 200 ul $\text{H}_2\text{O}$ Shake for 1 min. Centrifuge 5000 rpm for 2 min. Remove all $\text{CHCl}_3$ to scintillation vial. Rinse x 2 with new $\text{CHCl}_3$ . Transfer to vial. Evaporate $\text{CHCl}_3$ with $\text{N}_2$ . Add 500 ul Soluene 350. Digest for 2 hours at $50^\circ\text{C}$ .		

#### Kinetics of Glycine Uptake

The aim was to determine the rate of uptake of glycine by larvae in substrates of different concentrations. These were chosen to be 0,2  $\mu\text{M}$ , 0,4  $\mu\text{M}$ , 1,0  $\mu\text{M}$ , 2,0  $\mu\text{M}$  and 4,0  $\mu\text{M}$  of ( $1\text{-}^{14}\text{C}$ ) glycine. Larvae were prepared for the experiments by the method explained above for the time course experiments. Triplicate samples of 25 larvae were placed for one hour in 100ml of substrate for each of the

concentrations to be tested. After the required time, the larvae were removed and processed for scintillation counting.

#### Determination of Radioactivity in Larvae

Larvae resulting from each experiment were removed and washed in a 40  $\mu$ M sieve with 100  $\mu$ l SFAW. They were then placed in a beaker containing 100 ml SFAW at 28°C and allowed to stand for 10 - 15 mins. This procedure was repeated twice, each time larvae were thoroughly washed in the sieve. The larvae were then removed and placed on dry filter paper to remove any adherent fluid, then counted and placed in scintillation vials. They were then digested for two hours at 50°C with 1 ml of Tissue Solubiliser (Solubene 350). After cooling, 10 ml of Dimilume 30 scintillation fluid was added to each vial. All vials were kept in darkness overnight prior to counting on a Packard TriCarb 460 scintillation counter performing its own quench correction.

Knowing the total radioactivity of each sample, the number of larvae in each sample, and the specific activity of the isotope, uptake rates could be calculated as grams  $^{14}$ C-labelled glycine or glucose per milligram dry weight tissue. Results were expressed on a per milligram tissue basis rather than on a per larva basis so as to exclude variations resulting from individual weight variations in larvae from different batches.

### Oxygen Consumption

Oxygen consumption of the larvae was measured in a Gilson respirometer. About 100 larvae were placed in each of 12 reaction flasks in 25 ml of SFAW. In a side vial a piece of filter paper with KOH was added to absorb  $\text{CO}_2$  produced. Temperature was maintained at  $28 \pm 0,5^\circ\text{C}$  throughout the experiment. The prawns were not fed during the test. The larvae were acclimatised for two hours in the respirometer before readings were taken. At time zero, the flasks were closed and connected to a calibrated tube connected to a container filled with fluid. As the oxygen was consumed and the  $\text{CO}_2$  absorbed by the KOH, fluid moved up the tube. Readings were taken at hourly intervals and the volume of oxygen consumed calculated.

After a seven hour experimental period, the larvae in each flask were counted. As the dry weight per larva had been calculated by weighing a number of freeze dried larvae at the start of the experiment, it was possible to calculate the uptake of oxygen in  $\mu\text{l}$  per mg dry weight tissue.

### Bacterial Control

To monitor the effect of bacteria on the uptake of labelled substrate, a batch of larvae were preincubated in a solution containing 200 ppm of Penbritin, a wide-spectrum antibiotic for gram-positive and gram-negative bacteria. The larvae were preincubated for 18 hours prior to being placed in  $^{14}\text{C}$ -glycine labelled water at 0,4  $\mu\text{M}$ . The larvae

were placed in label for six hours and 24 hours and processed for scintillation counting in the manner described above.

An examination of about 100 larvae was performed on the scanning electron microscope to determine whether there was a bacterial flora on the surface or on any part of the larvae. The larvae were fixed in 2% gluteraldehyde, and dehydrated in alcohols after which they were critical point dried with CO<sub>2</sub>. The samples were then mounted on stubs and sputter coated with a 30nm layer of gold/paladium (ratio 60:40). The samples were examined at a magnification of 13 000 to give 2 um images. The surface of the larvae were, thus, carefully examined for bacteria.

## RESULTS

### Gut Clearance Time

On average the food took about 10 minutes to move through the gut from the moment the larvae had been fed. In some cases, however, it took up to two hours to clear the gut of any food ingested. It was, therefore, decided to allow 3 hours of gut clearance time in sterile water before the larvae were used for any DOC uptake experiments.

### Oxygen Consumption

The oxygen consumption of M. rosenbergii larvae was calculated to be  $10,15 \mu\text{l mg}^{-1} \text{ tissue hr}^{-1}$  with a standard deviation of 0,73 for 11 replicates (Figure 25).

### $^{14}\text{C}$ -Glycine

#### a) Time Course for $^{14}\text{C}$ -Glycine Metabolism

Figure 26 shows the uptake and tissue incorporation of  $^{14}\text{C}$ -glycine for a 46 hour period. The concentration of larvae used was generally between 0,5 and 1 larva  $\text{ml}^{-1}$  water. A calculation, based on the rate of uptake of substrate by the larva, reveals that the total uptake over the longest experiments did not reduce the substrate concentration by more than 5%. It may, therefore, be assumed that substrate concentration never became limiting.

The rate of uptake and incorporation in the tissue is fairly constant for the first seven hours after which incorporation rapidly decreases to almost zero. The rate of incorporation for the first five hours was calculated at 356,74 pg  $^{14}\text{C}$ -glycine  $\text{mg}^{-1}$  tissue  $\text{hr}^{-1}$ .

Figure 27 shows the production of  $^{14}\text{CO}_2$  over 24 hours. As the substrate used was (1- $^{14}\text{C}$ ) glycine, one molecule of substrate will produce one molecule of  $^{14}\text{CO}_2$ . It was, therefore, possible to convert all  $^{14}\text{CO}_2$  production values to an equivalent of  $^{14}\text{C}$ -glycine  $\text{mg}^{-1}$  tissue  $\text{hr}^{-1}$ .

It is important to note that, instead of a decrease in substrate uptake as in the tissue incorporation results, there is, in fact, a fairly sharp increase in the quantities of  $^{14}\text{C}$ -glycine that is taken up by the larvae and metabolised immediately to produce  $^{14}\text{CO}_2$ .

Figure 28 represents the combined values for substrate uptake representing the total amount of  $^{14}\text{C}$ -glycine absorbed by the larvae. Table 10 lists the values for the combined uptake of substrate, the rate of substrate uptake per hour and the percentage value which the substrate uptake represents of the total energy requirement of the larvae based on the following calculations. To metabolise 1ug of amino acid 1ul of oxygen is required (Stephens, 1975). Since the larvae consume 10,15 ul oxygen  $\text{mg}^{-1}$  tissue  $\text{hr}^{-1}$ , this can be converted to a requirement of 10,15 ug amino acid  $\text{mg}^{-1}$  tissue  $\text{hr}^{-1}$ . The following calculation can be made.

$$740 \text{ pg substrate} = \frac{100 \times 740}{10,15 \times 10^4} = 0,007\%$$

where  $1 \text{ ug} = 1 \times 10^4 \text{ pg}$

TABLE 10

Total  $^{14}\text{C}$ -Glycine Uptake in Starved Animals.

TIME (HRS)	TOTAL UPTAKE (In pg glycine $\text{mg}^{-1}$ tissue)	RATE UPTAKE/HR	% CONTRIBUTION TO ENERGY REQUIREMENT
1	740	740	0,007
3	3774	1258	0,01
15	45862	3057	0,03
24	134434	5601	0,06

Figure 29 represents the increase in the rate of uptake of glycine with time.

Table 11 gives the biochemical fate of  $^{14}\text{C}$ -glycine after absorption by starved M. rosenbergii larvae.

TABLE 11  
Biochemical Fate of  $^{14}\text{C}$ -Glycine.

Time		<u>Metabolic Fraction</u>	
		pg $^{14}\text{C}$ -Glycine mg <sup>-1</sup> tissue	Percentage of total uptake
3 Hour	Lipid	128,30	3,40
	Protein	526,68	13,96
	TCA Soluble	198,71	5,26
	CO <sub>2</sub>	<u>2920,00</u>	<u>77,38</u>
	Total Uptake	3773,69	100,00
24 Hour	Lipid	114,81	0,09
	Protein	2770,43	2,18
	TCA Soluble	708,42	0,55
	CO <sub>2</sub>	<u>123740,00</u>	<u>97,18</u>
	Total Uptake	127333,66	100,00

b) Kinetics of Uptake

Figure 30 shows the uptake of glycine by larvae exposed to substrate for one hour at concentrations of 0,2; 0,4; 1,0; 2,0 and 4,0  $\mu\text{M}$  solutions of  $^{14}\text{C}$ -glycine. The rate of uptake at 4  $\mu\text{M}$  solution is about three times that at 0,4  $\mu\text{M}$ , at which all the above experiments were conducted.

$^{14}\text{C}$ -Glucose

Figure 31 shows the time-course of uptake and tissue incorporation of (U- $^{14}\text{C}$ )-glucose by the larvae of M. rosenbergii. The same pattern is seen here as with that of uptake and tissue incorporation of 1- $^{14}\text{C}$ -

glycine. The rate of incorporation is constant at 232 pg  $^{14}\text{C}$ -glucose  $\text{mg}^{-1}$  tissue  $\text{hr}^{-1}$  for the first six hours after which it drops sharply to zero.

#### Algal Exudate

Figure 32 shows the time-course of uptake of  $^{14}\text{C}$ -labelled algal exudates. The concentration of the substrate was not known, thus it was not possible to determine the rate of uptake of labelled DOC by the larvae in milligram of substrate. The rate of uptake was constant for the duration of the experiment and calculated to be 19,05 DPM (decompositions per minute)  $\text{mg}^{-1}$  tissue  $\text{hr}^{-1}$ . The activity of the water was determined at the start of the experiment to be 6386 DPM  $\text{ml}^{-1}$  water (SD 125 DPM).

Table 12 lists the amino acids and their concentrations in algal exudate solution used for labelling experiments.

TABLE 12

Dissolved Free Amino Acids and their concentrations in a Phaeodactylum triconutum exudate solution similar to the labelled exudate solution used for DOC uptake experiments

Alanine	:	0,60uM
Arginine	:	0,79uM
Asparagine	:	1,30uM
Aspartic acid	:	1,01uM
Cystine	:	1,74uM
Glycine	:	0,40uM
Histidine	:	0,46uM
Serine	:	0,41uM
Total		6,71uM

### Antibiotic and Bacterial Control

Antibiotic experiments for six hours and 24 hours show a reduction in uptake of glycine by about 35% in both cases.

Scanning electron microscope (SEM) examination showed that very few bacteria adhere to the surface of the animals. No bacteria were found on the carapace, abdomen and appendages (Figures 33 and 34). Only a few bacteria, mainly rods, were found on some of the setae on the telson and on the antennules of some of the larvae.

## DISCUSSION

Though the uptake of dissolved organic carbon in marine invertebrates is now a well documented fact, doubt still remains concerning the crustacea. Anderson and Stephens (1969) showed uptake of  $^{14}\text{C}$ -glycine in a number of crustacea. However, when the animals had been pretreated with antibiotics the uptake was reduced to insignificant levels. They concluded that the bacterial epiflora was responsible for the removal of the labelled substrate and that there was no evidence for the accumulation or assimilation of small organic compounds by the small crustaceans examined. Castille et al (1979), Lawrence (1973) and Lawrence et al (1975), using antibiotics in the medium found that bacteria were the cause of most of the uptake of labelled DOC in crustacea. Sorokin and Wyshkwarzev (1973), however, reported the uptake, by small crustaceans, of labelled algal hydrolysates in the presence of streptomycin (100 mg/L).

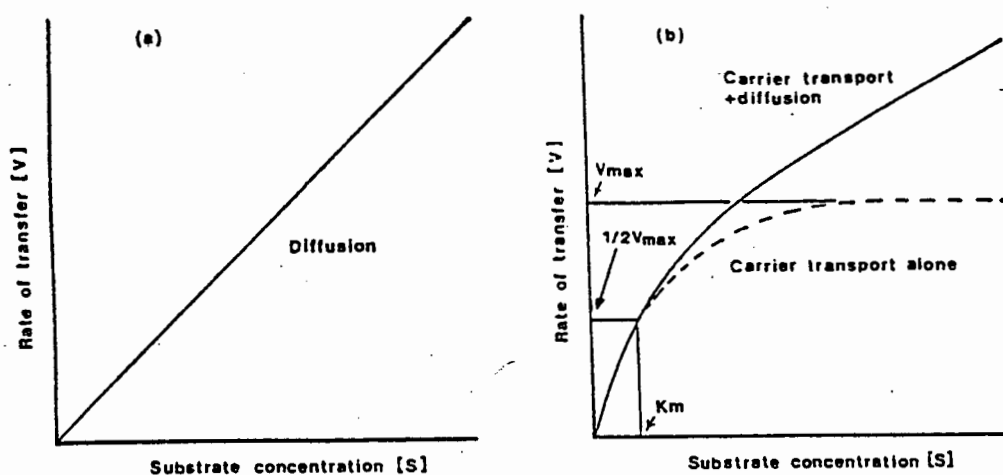
Mitchison and Cummins (1966) have shown that antibiotics can block the mechanism of absorption of organic molecules by the membranes in sea urchin. This, therefore, questions the validity of the results of experiments where antibiotics have been used to eliminate the action of bacteria and to demonstrate the impermeability of the animals to dissolved organic compounds.

Pavillon and Vu Tan Tue (1981), using histoautoradiographic determination, clearly demonstrated the uptake of DOC by the eggs of Artemia salina. Earlier, Pavillon et al (1980), by the same

techniques, had shown that in 10 day old Artemia marked substances had penetrated into the intestinal wall. They state: "There is no doubt that an active absorption of amino acids dissolved in the medium by the eggs and nauplii of Artemia occurs during the first 48 hours."

The examination of larvae by scanning electron microscope has shown an extremely limited epiflora of bacteria. This seems to conform to the findings of Aquacop (1977b) who find bacteria growing on the larvae only when these become unhealthy. They do not report the presence of bacteria on healthy larvae. It was, therefore, considered that bacteria did not have a significant effect on the DOC uptake values during our experiments.

In the study of uptake of dissolved organic substances by marine larvae, the selective absorption of labelled substrate such as amino acids, glucose and fatty acids was examined (Pavillon, 1981). The prevalent theory is that absorption can be due (i) to diffusion through the tissues, which is a purely physical phenomenon controlled by differences in concentration (Figure 35a); or (ii) to active transport by a carrier molecule (Figure 35b) (Stewart, 1979).



**FIGURE 35:** Graphic representation of entry of solute molecules into cells: a, diffusion - when the rate of transfer increases in a linear relationship with concentration; b, broken line, carrier transport alone; solid line, combination of carrier transport and diffusion; this plot may be converted to one for carrier transport alone by subtracting the diffusion component from the values used to draw the solid line;  $V_{max}$  is the maximal rate of transfer reached in the presence of a saturating concentration of substrate and  $K_m$  is the substrate concentration =  $1/2V_{max}$  (from Stewart, 1979).

In the case of active transport the absorption of substrate and the saturation of carriers is reminiscent of the law of Michaelis-Menton on the rate of enzymatic reaction (Stewart, 1979).

Figure 30, illustrating the kinetics of uptake of  $^{14}C$ -glycine at different concentrations is very similar to Figure 33b suggesting carrier transport. It is possible that with concentrations higher than 4,0  $\mu M$  the rate of uptake would become saturated to a maximum value. Figure 30, however, clearly illustrates that the absorption of DOC by the larvae of M. rosenbergii involves a carrier transport

mechanism which might work alone or in combination with a diffusion process of uptake.

The results obtained for the time-course study for tissue incorporation of  $^{14}\text{C}$ -glycine and  $^{14}\text{C}$ -glucose (Figures 26 and 31) are very similar and are characteristic of active transport. Saturation starts intervening in both cases from about six hours.

It is possible, though, that the process of uptake and tissue incorporation of dissolved organic matter follows a more complex process. Marine invertebrates are known to contain large pools of free amino-acids (Stewart, 1979). Starvation may significantly affect this pool. Fair and Sick (1982) in a five day starvation experiment on M. rosenbergii intermoult adults, found a significant decrease in the concentrations of all free amino acids in the serum of the prawn, with the exception of glycine. Similarly, Shick (1975) found that, on starvation, the level of most components of the free amino acid pool decreased, in the polyps of the coelenterate Aurelia aurita, with the exception of glycine, beta-alanine and taurine whose levels increased.

M. rosenbergii larvae are known to have a much higher metabolic rate than the adults (Stephenson and Knight, 1980; Nelson et al, 1977). As reserves are small in the fast growing larvae (Stephenson and Knight, 1980), it is likely that the depletion of the free amino acid pool, at starvation, will be rapid. It is suggested here that the drop in tissue incorporation of glycine on starvation is initially not due to a saturation process but is, rather, the result of the depletion of the free amino acid pool. In the early stages, when the pool is still

at its normal level, protein synthesis, and as a result, labelled glycine incorporation, can take place. As the amino acid pool becomes depleted, protein synthesis take place at a decreasing rate until a point is reached when there are no more free amino acids available with which the  $^{14}\text{C}$ -glycine can form proteins and tissue incorporation no longer takes place.

It is possible that a similar mechanism controls and limits the uptake of glucose which is also seen to reach a saturation point (Figure 31).

This is substantiated by the results obtained with the uptake of algal exudates (Figure 32). The total free amino acid concentration alone was in excess of 6  $\mu\text{M}$  (Table 12), far above the 0,4  $\mu\text{M}$  and 0,8  $\mu\text{M}$  used for the glycine and glucose experiments respectively. Though it was not possible to determine, within the scope of these experiments, which fractions of the exudates were labelled, it is reasonable to assume that it was not restricted to a single compound. It is also reasonable to assume that the uptake of DOC by the larvae was not restricted to only a few compounds, but rather covered a wide range of substances. The uptake of labelled compounds was constant throughout the experiment. The possibility that saturation had not taken place by the end of the experiment because of the scarcity of some substances was discarded. The free amino acid concentration in the culture was in excess of 6  $\mu\text{M}$  and the glycine concentration was 0,4  $\mu\text{M}$ , that at which the pure glycine experiments were performed.

The allocation of  $^{14}\text{C}$ -glycine for respiration increases with starvation from 77% of the total uptake at 3 hours to 97% at 24 hours. Manahan (1983) finds that 12,5% of the total glycine absorbed by Crassostrea gigas veliger larvae is respired at 10 minutes and increases to 33,7% at 100 minutes. Shick (1975) also finds  $^{14}\text{CO}_2$  production of Aurelia aurita polyps in  $^{14}\text{C}$ -glycine enriched medium is, in starved animals, double that of non-starved polyps. Schlichter (1973) found that 80% of the glycine and serine taken up by Anemonia sulcata was respired by the animal. Schlichter (1975) found that glucose was rapidly respired, with only about 10% of the total glucose absorbed being incorporated in the tissue.

With starvation, the rate of uptake of glycine for respiration (Figure 27) and thus, the total rate of uptake (Figure 28) increases in the larvae of M. rosenbergii. The rate of increase, however, appears to be constant (Figure 29). These results differ from those of Manahan (1983) and Shick (1975). Shick (1975) found that the total rate of uptake of glycine by Aurelia aurita polyps did not change with starvation. Manahan (1983), on the other hand, found that the total uptake of glycine by Crassostrea gigas larvae dropped from 4,23 pg/larva/minute at 10 minutes exposure to 2,83 pg/larva/minute at 100 minutes in the pediveligers and from 2,56 pg/larva/minute at 10 minutes to 2,07 pg/larva/minute at 100 minutes in veligers. Other similar information is not available in the literature. As all these experiments were run in different conditions, it is difficult to draw comparative conclusions from their differences in rates of uptake with starvation. Our results, however, seem to indicate that, with

starvation, the mechanism for the uptake of dissolved organic compounds is activated and becomes increasingly important with increasing starvation.

Lee and Bada (1977) estimate the dissolved free amino acid (DFAA) concentration in the open ocean to be 0,4  $\mu\text{M}$ , but that of coastal waters, which are similar to estuarine waters, to be ten times higher. Hobbie et al (1968) report a DFAA concentration in the York River estuary, Virginia, to be about 0,4  $\mu\text{M}$ . Crawford et al (1974) reported similar concentration of DFAA in the Pamlico River estuary, North Carolina. The DFAA, however, were only about 0,2% of the total dissolved organic carbon in the water. Stephens (1967) reports a DFAA concentration of between 10 - 100  $\mu\text{M}$  in inshore waters. It seems, therefore, that the DFAA in inshore and estuarine systems has a considerable range of concentrations.

The glycine uptake experiments were performed at a concentration of 0,4  $\mu\text{M}$ . The kinetics experiment (Figure 30) showed that at 4  $\mu\text{M}$  the rate of uptake and incorporation of glycine was three times as high. The algal exudate uptake experiments, performed at a DFAA concentration in excess of 6  $\mu\text{M}$  showed a constant rate of uptake of label throughout the experiment. Glycine is only one of the many small molecular weight compounds in solution in sea and estuarine waters. Other compounds include hormones, vitamins, pterines, peptides, fatty acids and amino acids (Pavillon, 1976b). Many of these compounds are independently transported and assimilated by marine invertebrates (Stewart, 1979). Though the energy value of glycine absorbed by the

larvae of M. rosenbergii at 0,4 uM concentration is very small (Table 10), it is possible that the combined contribution from the uptake and metabolism of a broad spectrum of dissolved organic compounds, which would be found in the natural waters of estuaries where M. rosenbergii larvae grow, and in the algal rich larval culture systems in M. rosenbergii hatcheries, could make a greater contribution to the larval energetics than is indicated by the results shown here.

In conclusion, this work has clearly demonstrated that a mechanism for uptake of DOC by the larvae of M. rosenbergii exists. At this stage, it appears that the energy value of the DOC for the nutrition of larvae is small. However, vitamins and other compounds may play a significant role as growth factors promoting larval development. This may, also, be the case in the reports by Maddox and Manzi (1976) and Manzi and Maddox who show that algal supplements significantly increase larval survival and growth rates in the larvae of M. rosenbergii.

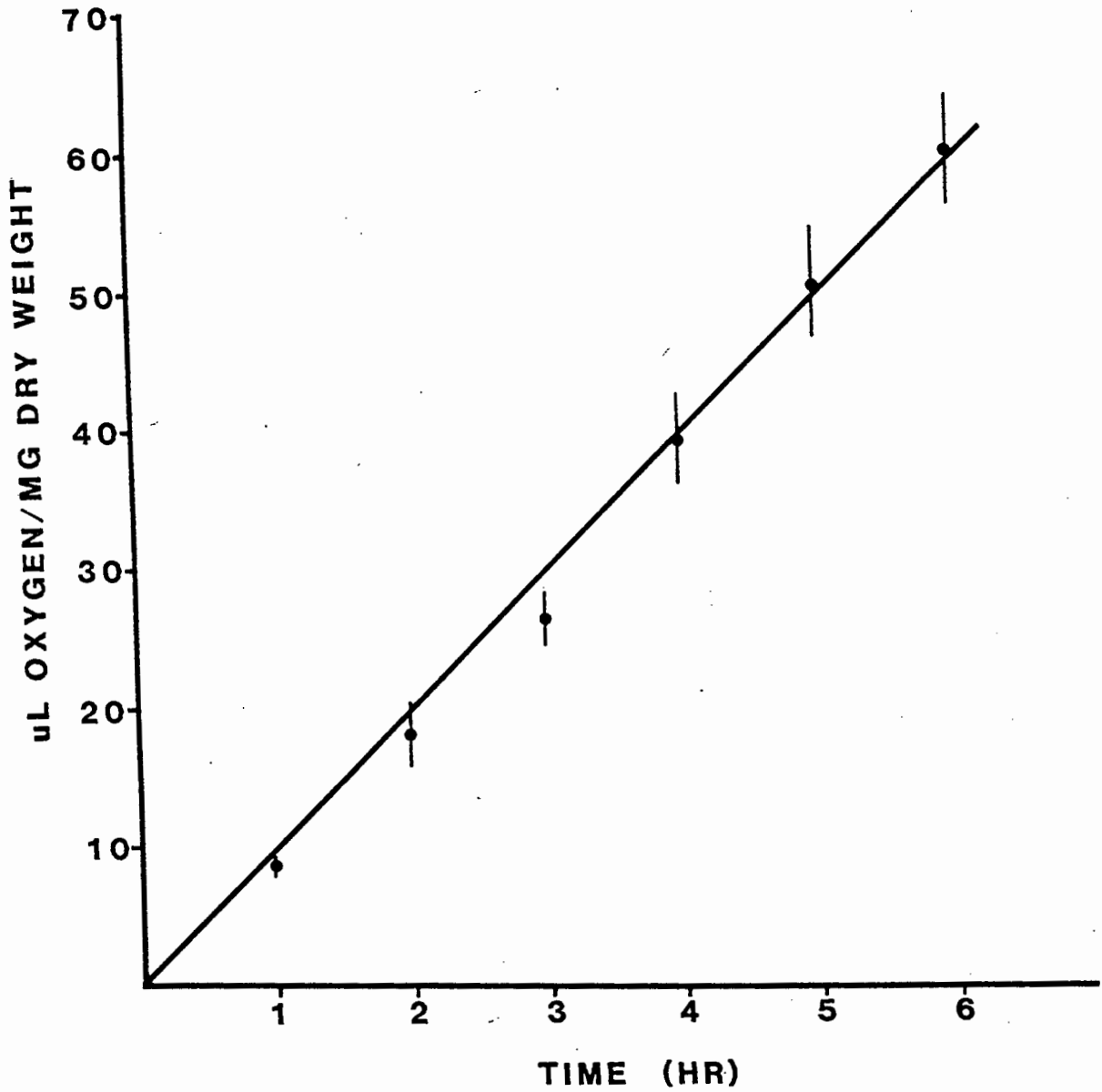


Figure 25: The oxygen consumption of LS2 larvae.  $R^2=0,97$ .

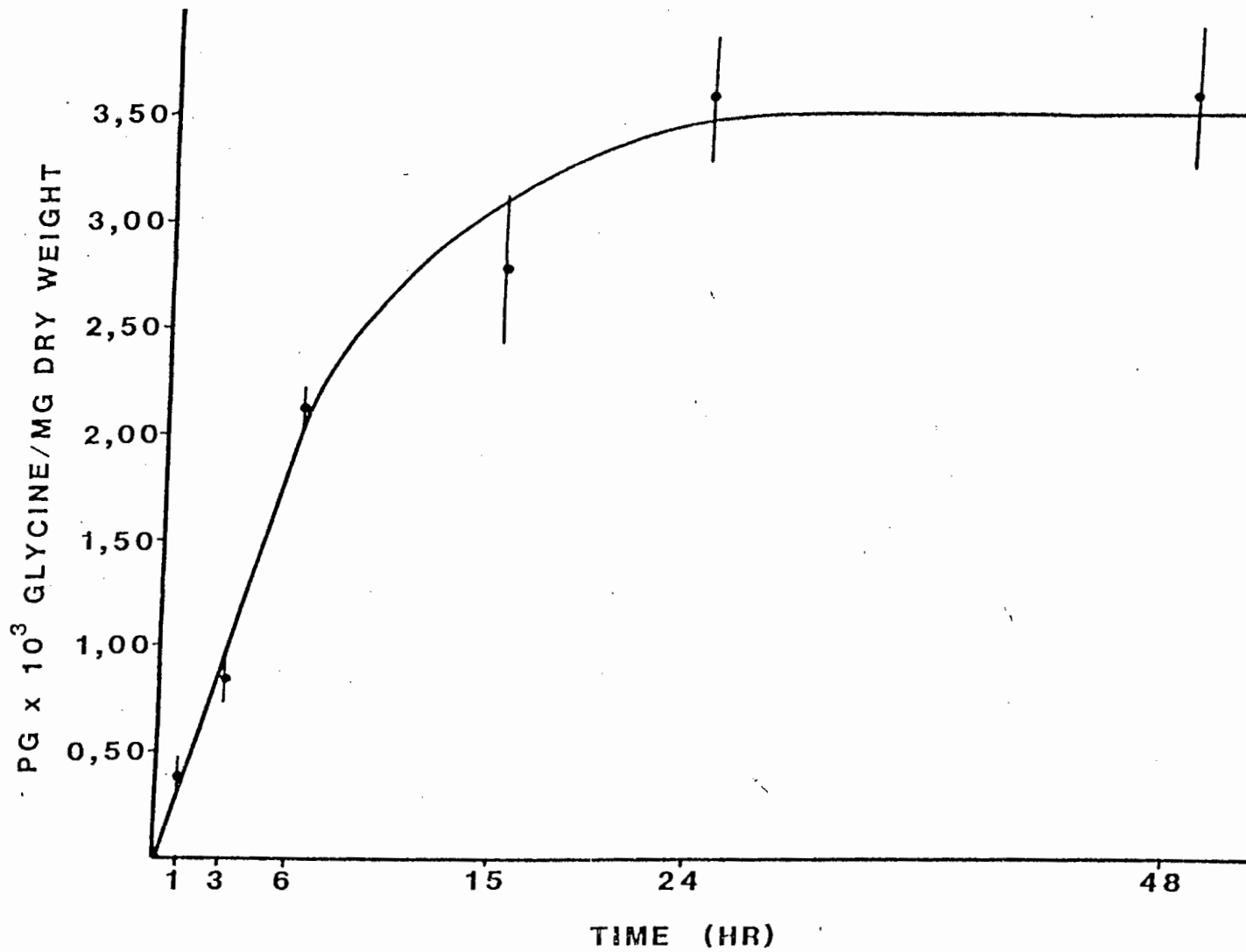


Figure 26: Time course of  $^{14}\text{C}$ -glycine uptake and tissue incorporation of label  $^{14}\text{C}$ -glycine concentration = 0,4  $\mu\text{M}$ . All data points are mean  $\pm$  S.D. for n = 3.

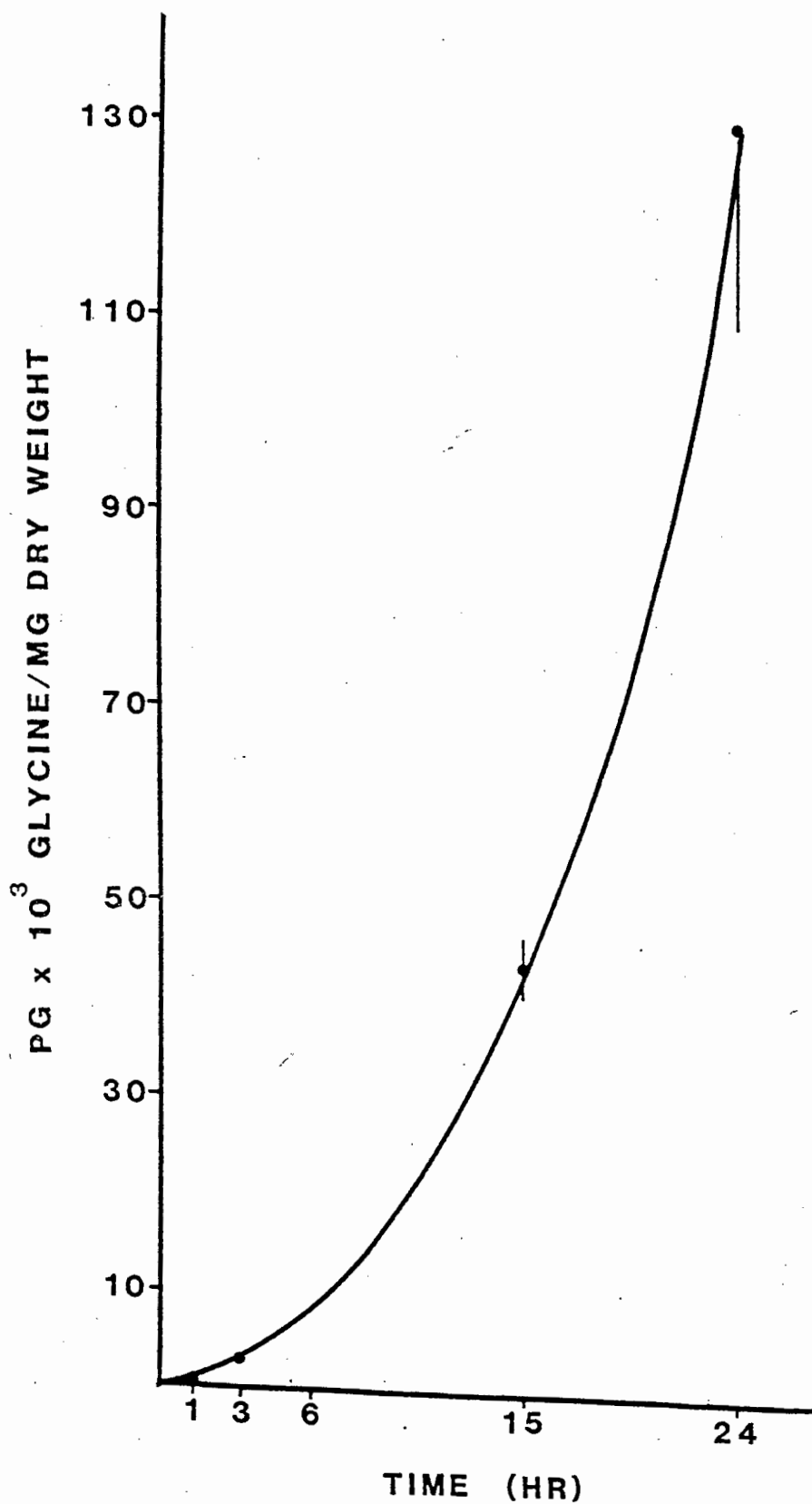


Figure 27: Time course of  $^{14}\text{C}$ -glycine uptake and respired  $^{14}\text{CO}_2$ .  $^{14}\text{C}$ -glycine concentration = 0,4  $\mu\text{M}$ . All data points are mean  $\pm$  S.D. for  $n = 3$ . Where no bar S.O. are shown, the bar lies within the graphical presentation of the point.

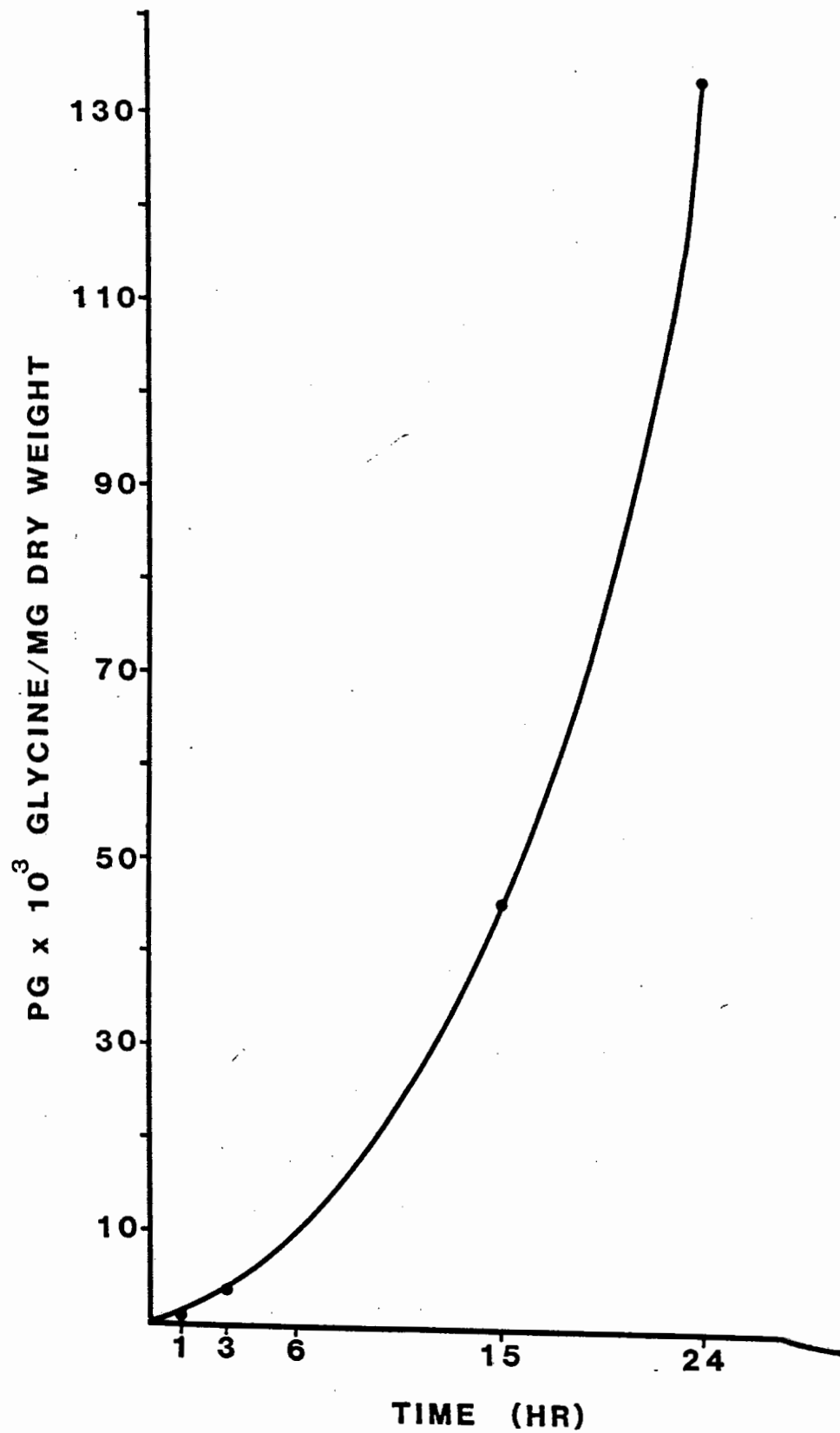


Figure 28: Total  $^{14}\text{C}$ -glycine uptake by larvae combining the tissue incorporated  $^{14}\text{C}$ -glycine and the  $^{14}\text{C}$ -glycine respired as  $^{14}\text{CO}_2$ .

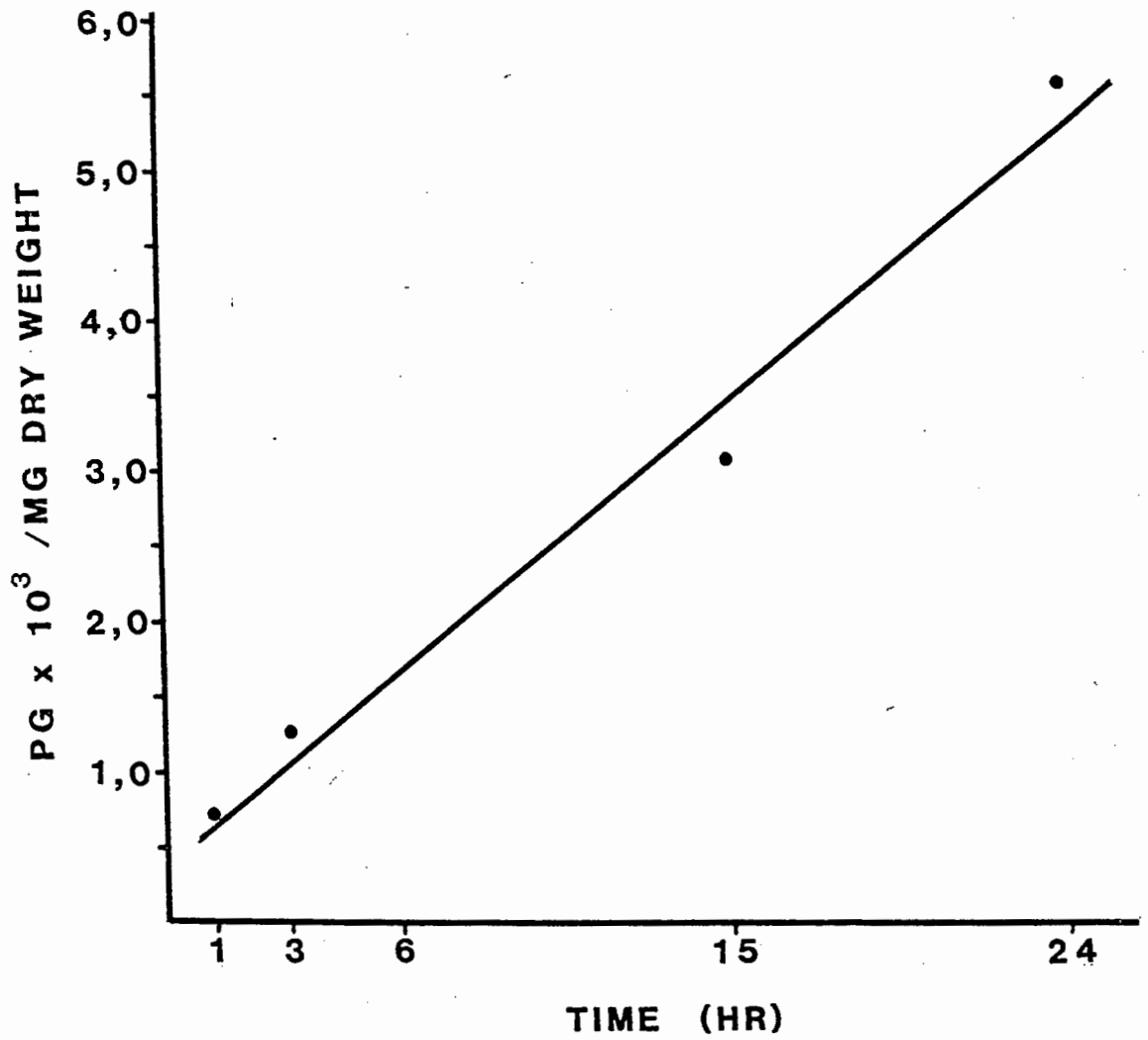


Figure 29: Increase in the rate of total uptake of <sup>14</sup>C-glycine in larvae. Correlation coefficient  $R^2 = 0,98$ .

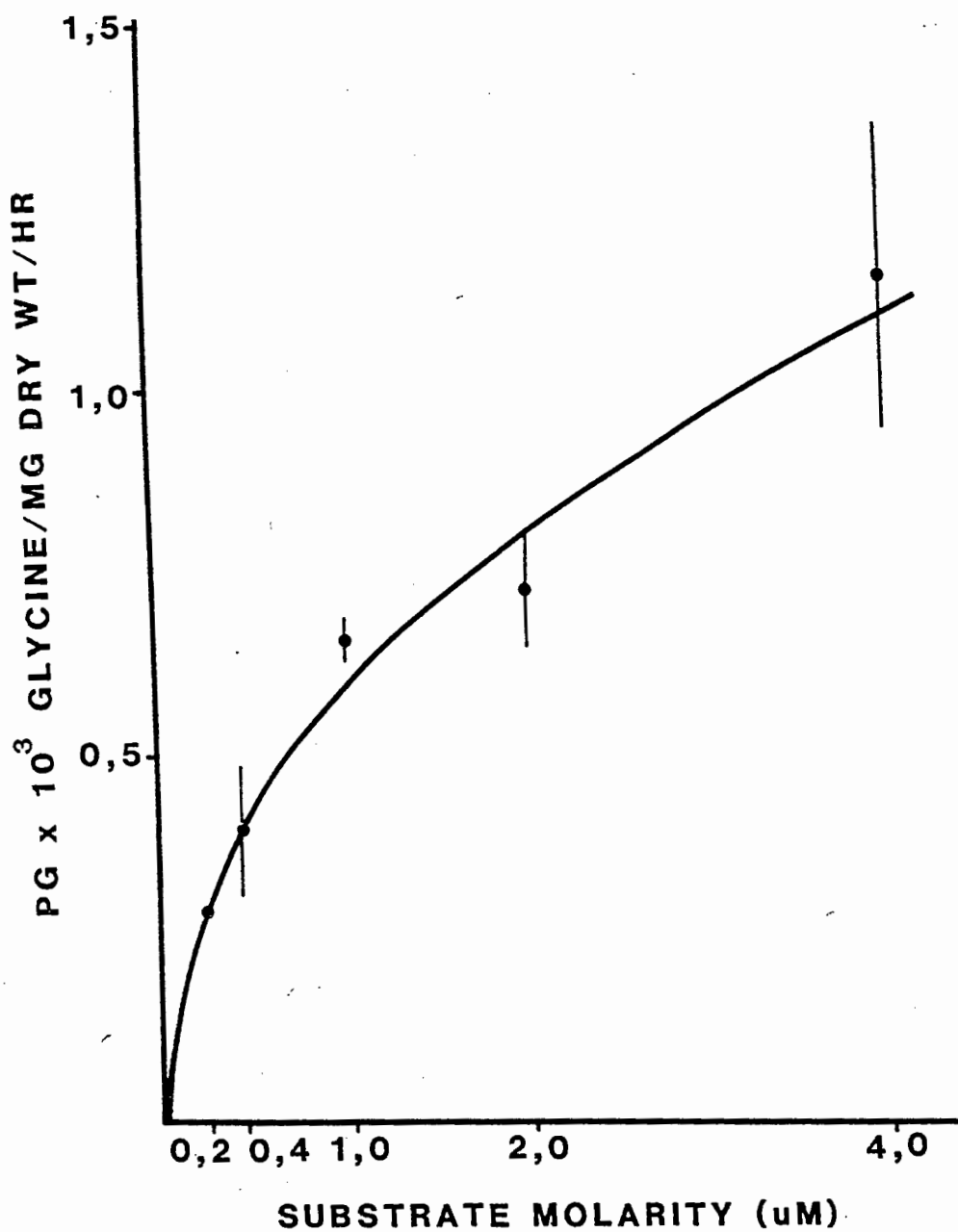


Figure 30: Kinetics of <sup>14</sup>C-glycine uptake at different substrate concentrations. All data points are mean ± S.D. for n = 3. R<sup>2</sup> = 0,98.

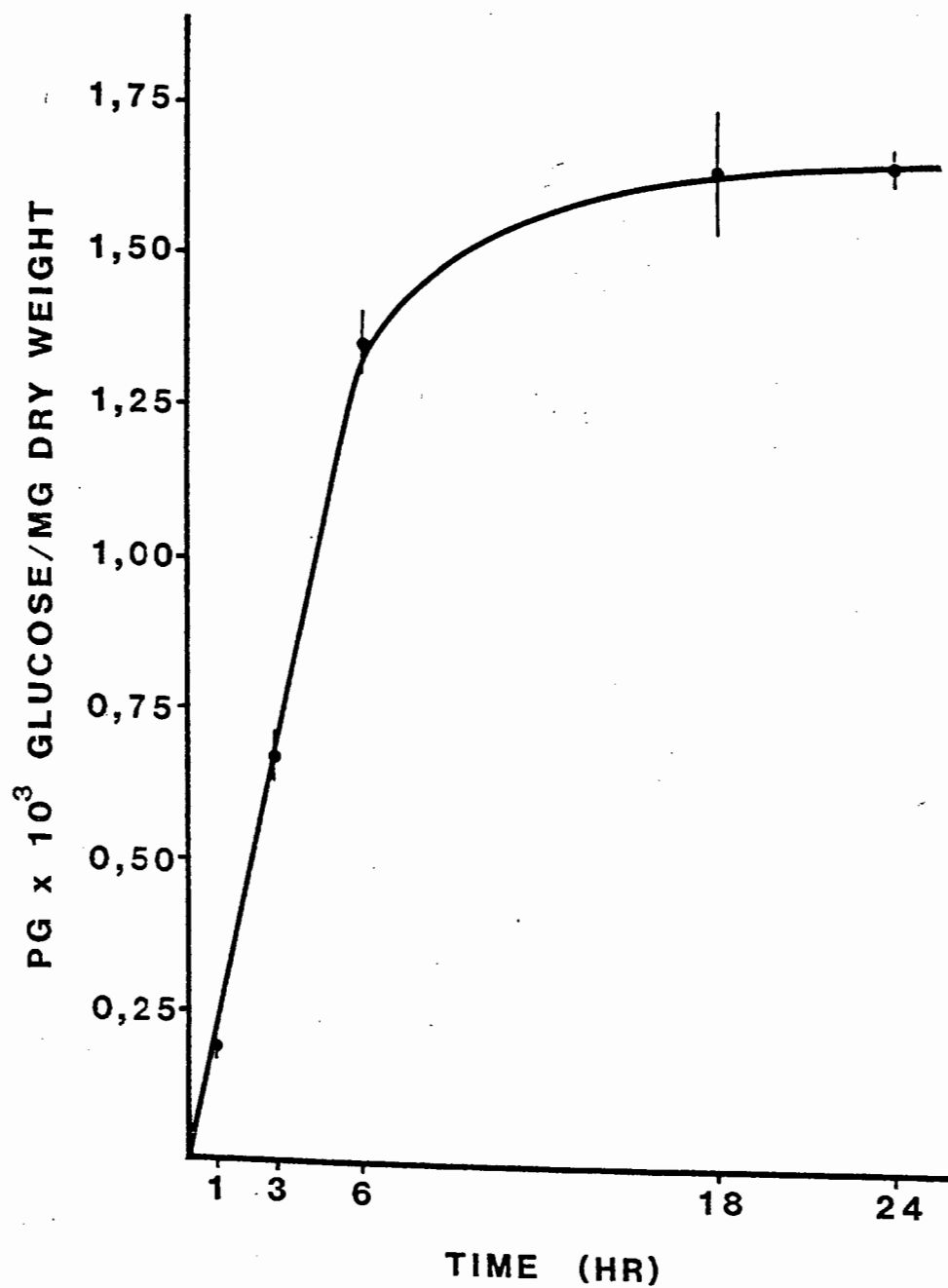


Figure 31: Time course of  $^{14}\text{C}$ -glucose uptake and tissue incorporation of label.  $^{14}\text{C}$ -glucose concentration = 0,8  $\mu\text{M}$ . All data points are mean  $\pm$  S.D. for  $n = 3$ .

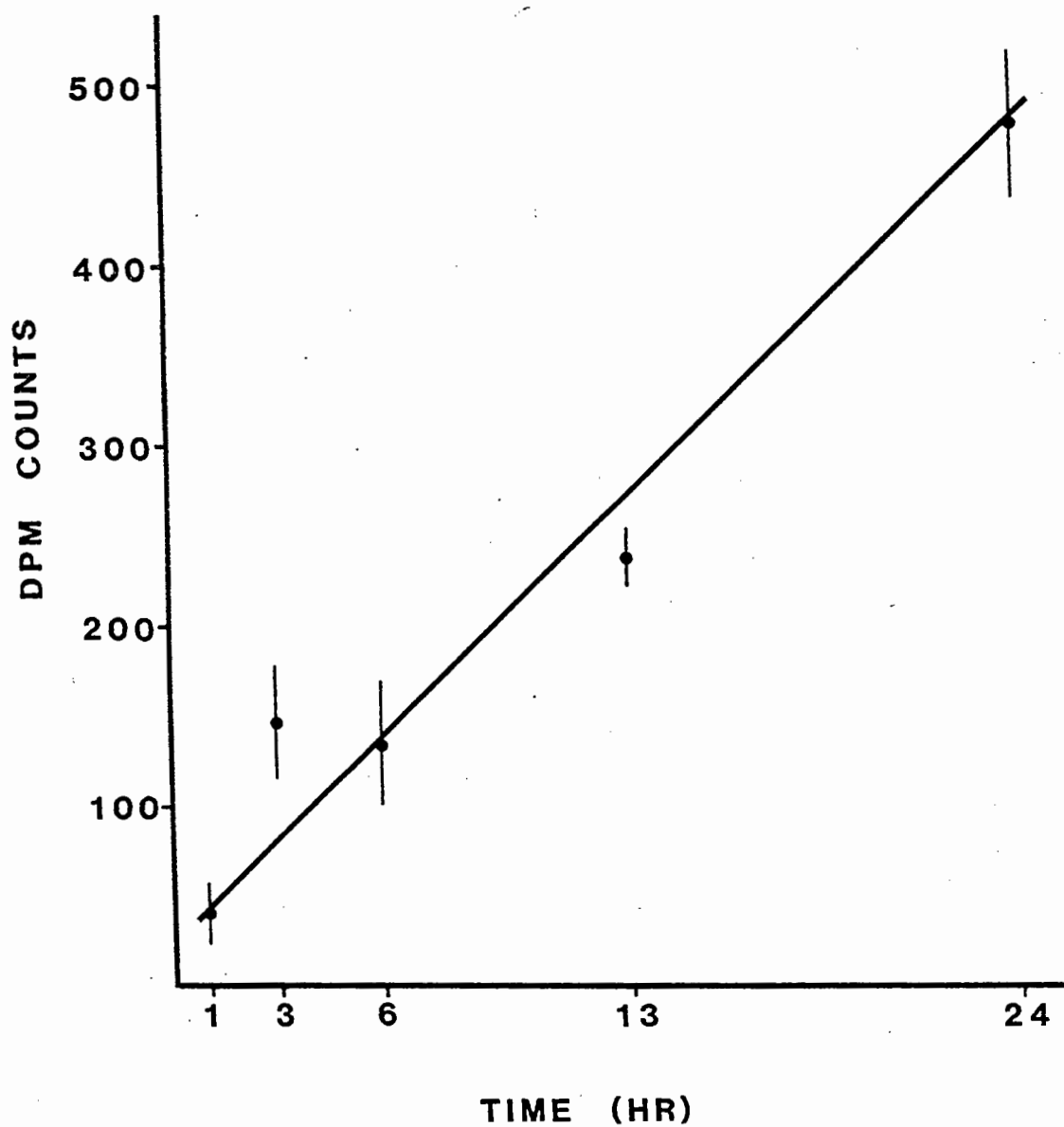


Figure 32: Time course of  $^{14}\text{C}$ -labelled algal exudate uptake and tissue incorporation. Exudate concentration was not determined. All data points are mean  $\pm$  S.D. for  $n = 3$ .

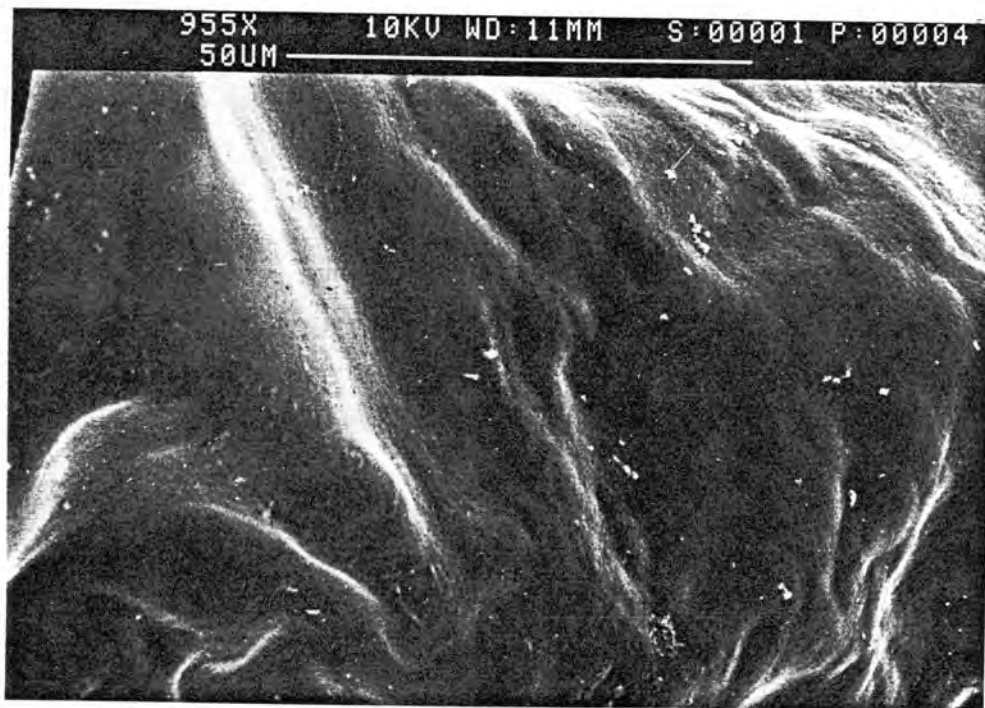


FIGURE 33: Scanning electron microscope photograph of larval carapace showing conspicuous absence of epiphytic bacterial flora.

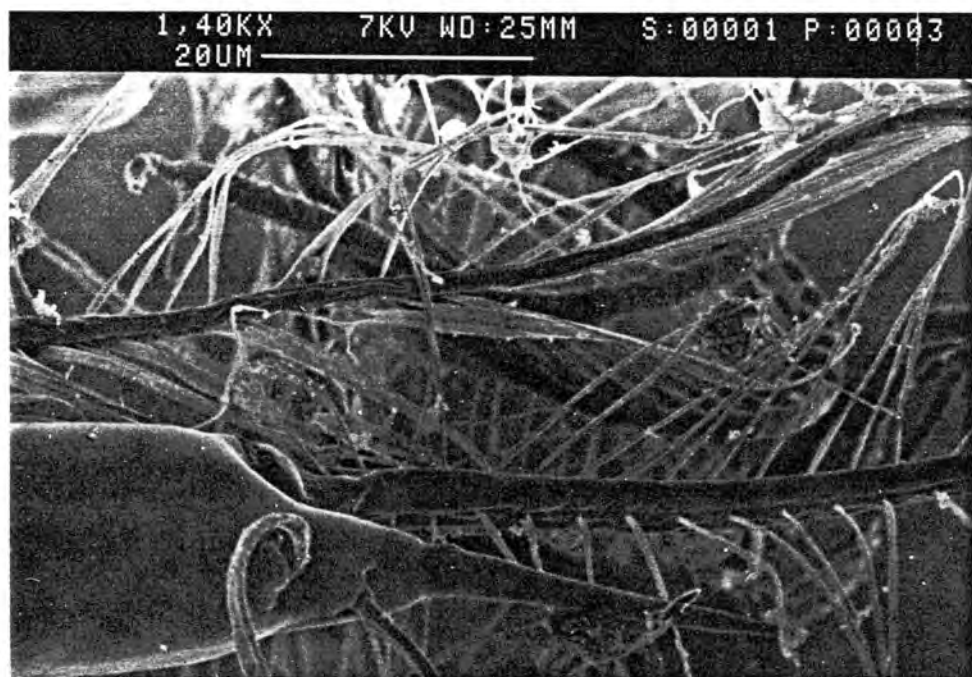


FIGURE 34: Scanning electron microscope photograph of antennae of larva showing absence of epiphytic bacterial flora.

GENERAL CONCLUSION

In this study, an attempt has been made to determine whether phytoplankton has a nutritive value for the larvae of M. rosenbergii. The study of the lipids during the larval cycle has shown that there are no significant differences in the composition of the lipid classes and the fatty acids of the larvae, whether these larvae were reared in "green water" or in clear water. A reason for this could be that the lipid content of the food given to the larvae, mainly in the Artemia salina instar I nauplii (19 - 23% of the dry weight (Benyts et al, 1975)) is so high that these dominate the composition of the larval lipids. This would be in accordance with the findings of Sandifer and Joseph (1976) who showed that the fatty acid composition of juvenile M. rosenbergii changed with that of the diet. It was, therefore, concluded that phytoplankton had no significant effect on the composition of lipids in M. rosenbergii larvae.

In the study on the role of dissolved organic substances, it was shown that a mechanism for the uptake of these substances does exist in the prawn larvae. It was also shown, however, that the dissolved organic compounds are not of major importance as a source of energy in the nutrition of the larvae. The possibility that algal exudates act as growth factors and that these have a qualitative rather than a quantitative value in the nutrition has been discussed.

The nutritional role of phytoplankton in the culture of M. rosenbergii larvae remains unclear. On the basis of the present study one can

conclude that phytoplankton and its exudates are not an important energy source for these larvae. At this stage, however, one cannot discard the possibility that phytoplankton may play some type of more specific role in the nutrition of larvae, particularly where vitamins and hormones are concerned. Very little work has been done in this field, and that mainly on Artemia, Euphansia krohnii and the urchin Arbacia lixula nauplii ad pluteus larvae (Pavillon, 1976a; 1976b; 1977).

To determine the precise extent to which phytoplankton may have nutritional importance for the larvae of M. rosenbergii, future research will have to be directed at determining the role that the DOC (in particular vitamins and hormones), play in the growth, development and energetics of the larvae and in the enhancement of food assimilation these components might provide. Research should be directed towards identifying specific compounds in algal exudates and their action on the larvae.

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APPENDIX 1MICROANALYTICAL QUANTIFICATION OF TISSUE LIPIDS  
(Holland and Gabbot, 1971)

2 - 3 mg of the freeze-dried sample was weighed and homogenised in an all-glass homogeniser with 100 ul distilled water. This was transferred to a small Pyrex test tube. The homogeniser was rinsed with 100 ul water which was added to the contents of the test tube. 3 500 ul methanol and 250 ul chloroform were added to the test tube, mixed thoroughly and allowed to stand at 4° C for 10 minutes. The sample was then centrifuged at 800 g for 10 minutes and the supernatant collected and transferred to a clean Pyrex tube.

250 ul chloroform and 250 ul water were added to the supernatant and the contents of the tube well mixed and allowed to stand at 4° C for five minutes after which it was centrifuged for 10 minutes at 800 g. The bottom phase consisting of chloroform and dissolved lipids was transferred to a clean tube and evaporated to dryness with a flow of N<sub>2</sub> gas. 500 ul of chloroform was then added to the lipid residue and mixed thoroughly.

- i) Total Lipid Content : 3 x 50 ul aliquots of the chloroform/lipid mixture were placed in small Pyrex test tubes and evaporated to dryness with a flow of N<sub>2</sub>.
- ii) Neutral Lipid Content : To the remainder (350 ul), 500 ul chloroform was added together with about 10 mg of activated silicic

acid. This was well shaken and allowed to stand for 15 minutes at 4° C. The silicic acid absorbed all the phospholipids leaving the neutral lipids in solution in the chloroform. The tube was then centrifuged at 800 g for 15 minutes and 3 x 200 ul aliquots of the chloroform were placed in small Pyrex test tubes and evaporated to dryness with N<sub>2</sub> gas.

#### Lipid Determination

Standards : 5, 10, 20 and 40ug tripalmitin in 50ul chloroform. All standards tested in triplicate.

To each total lipid, neutral lipid and standard replicate were added : 500 ul AR. H<sub>2</sub>SO<sub>4</sub> which was well mixed and heated for 15 minutes at 200° C. The tubes were then cooled in water. 1,5 ul H<sub>2</sub>O was then added, mixed well and cooled. This was then read on a Beckman 25 spectrophotometer at 375 nm in a 2 cm cuvette.

The values for the standards were measured and plotted against concentration on graph paper and the sample spectrophotometric values could thus be read on the graph and the corresponding lipid concentrations obtained. Thus, the quantity of total and neutral lipids in the original sample could be calculated.

APPENDIX 2TOTAL LIPID EXTRACTION  
(Bligh and Dyer, 1959)

2 - 3 grams of a frozen sample were transferred to a glass homogenising tube to which water, chloroform and methanol were added in the following volumes : 4 ml H<sub>2</sub>O : 5 ml chloroform : 10 ml methanol. The sample was then homogenised under a nitrogen atmosphere for 2 - 3 minutes or until it was completely homogenised. In the above proportions the solvents are totally miscible. 5 ml of chloroform was then added and mixed for about 30 seconds, again under a nitrogen atmosphere. Finally, 5 ml of water was added and mixed for a further 30 seconds. The tube was then centrifuged for 10 minutes at 1500 rpm. In the new proportions the liquids are no longer miscible and the chloroform will separate from the water and methanol mixture. The chloroform sinks to form the supernatant of the biphasic mixture. The total lipids, at that stage are dissolved from the tissue into the chloroform and the methanol/water a protein rich scum accumulates on centrifugation.

The chloroform was then pipetted out and transferred to a vial for storage. An equal amount of chloroform was again added to the water and methanol, mixed well and centrifuged again at 1500 rpm for 10 minutes. The chloroform, which contained any residual lipids left in the protein scum, was then added to the first chloroform extract. This was evaporated with nitrogen gas to remove any traces of water and redissolved in chloroform and stored at -13° C in amber vials in a

nitrogen atmosphere to prevent oxidation of the lipids. Anhydrous silicic acid was added to the vials to remove any trace of moisture from the sample.

APPENDIX 3THIN LAYER CHROMATOGRAPHY

The TLC plates have a 0,25 mm thick layer of silica gel absorbent spread evenly on the glass. The brand used was Merck 20 cm x 20 cm plates. The lipid extracts to be analysed were applied as 5 mm x 1 mm bands about 2 cm from the lower edge of the chromatoplate (the "origin") using a Camag Linomat III spot applicator. The individual bands were 1 cm apart and the two outermost bands at the origin were 2 cm from the edges of the plates.

The plates were then placed in a closed chromatography tank filled with developing solvent to a depth of about 1 cm. The solvent was placed in the chamber at least one hour before the immersion of the chromatoplate to allow saturation of the atmosphere in the chamber with solvent. This is necessary to ensure uniform migration of the solvent on the plates.

The solvent used was a mixture of petroleum ether : diethyl ether : acetic acid in the proportions 90:10:1 v/v/v. This mixture is used specifically for the migration and separation of neutral lipids.

The developing solvent was then allowed to rise until it reached 3 - 4 cm from the top edge of the plate for the first development. The plates were then removed, air dried and replaced in the chamber for a second migration (second development) to obtain a better separation of

the lipids. The developing solvent carries the compounds present in the lipid fraction up with it as it migrates up the plate. The different lipid fractions will stop migrating up the plate at different positions on the plate. This distance depends on the chemical structure of the compounds and its polarity and on the composition of the developing solvent. In this way different lipid classes move to different levels to form "spots" or "bands". The compounds are usually colourless, thus the chromatoplates are finely and uniformly sprayed with a special reagent in order to detect the spots. In the present case 40%  $H_2SO_4$  was used and heated to  $180^\circ C$  for about 15 minutes to char the lipids leaving a series of brown carbon bands on the plates (Plate 1). The lipids should never be heated to more than  $200^\circ C$  on the chromatoplates to avoid loss by evaporation (Privett, 1965).

The spots represented the different lipid compounds that had migrated up the plates. For the identification of these compounds, cocktail was applied to the chromatoplates when the lipid samples were applied. The individual lipids in the cocktail had been previously tested for migration position. Thus the different lipid fractions in the samples could be identified by comparison of migration distance from the origin.

Quantitative analysis of the lipid spots was done by photodensitometric measurement of transmitted light using a Vitatron TDL 100 Universal Densitometer using tungsten light and a beam 0,1mm in diameter. The instrument was adjusted so that it gave a reading of

100% transmission when the light was passed through the plate between the spots and zero percent transmission when the shutter in front of the photocell was closed. The passage of each spot over the slit gave a peak of optical density values (Privett, 1965). An integrator attached to the densitometer calculated the area of each peak allowing percentage values for the different lipid classes in each sample to be calculated.

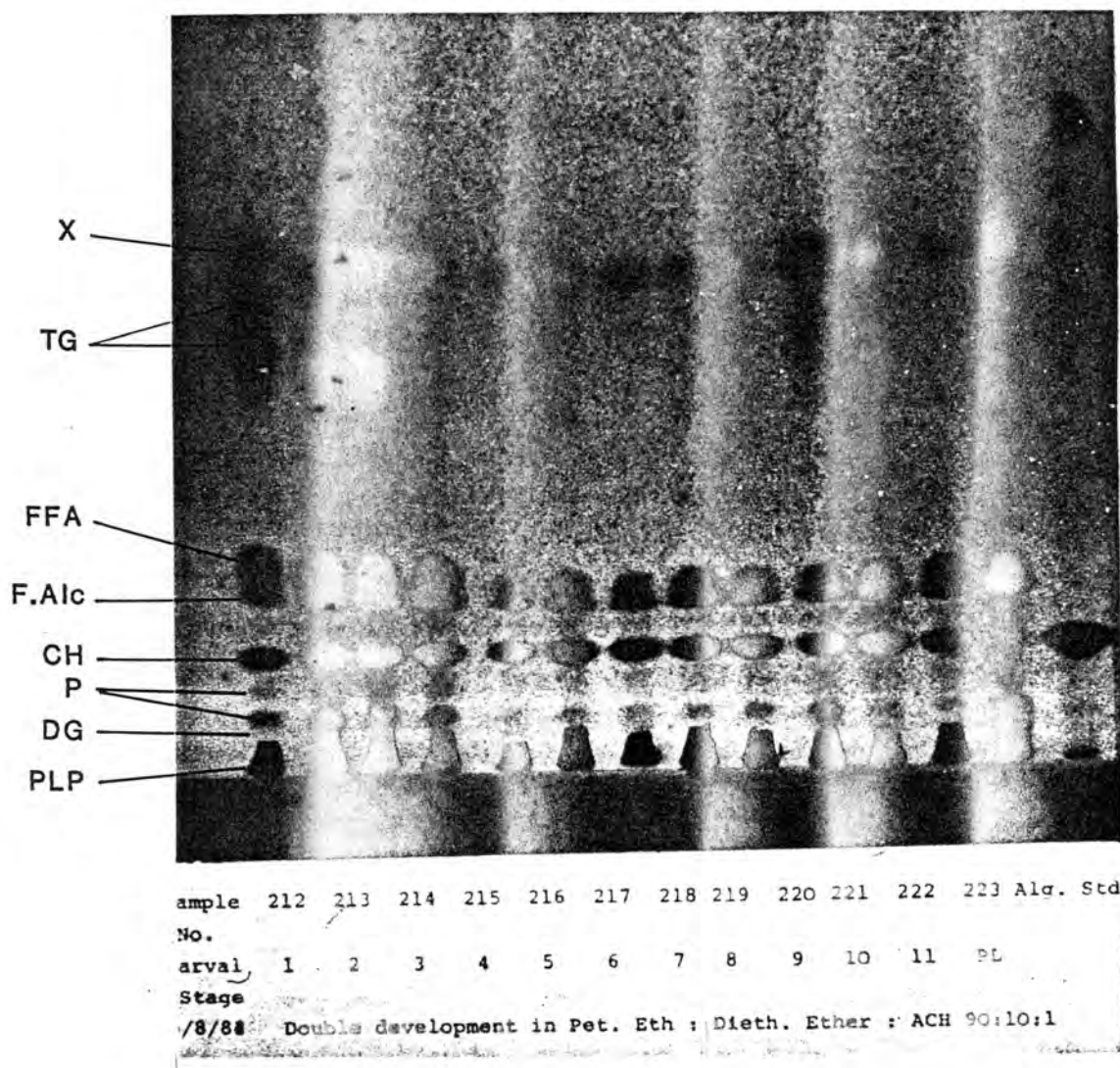


PLATE 1: Thin Layer Chromatography of Lipids in the Larvae of *M. rosenbergii* (X: Unknown, TG: TRIacylglycerols, FFA: Free Fatty Acids, F.Alc: Free Fatty Alcohols, CH: Cholesterol, P: Pigments, DG: Diacylglycerols, PLP: Phospholipids and Pigments).

APPENDIX 4METHYLATION OF LIPIDS  
(Morrison and Smith, 1964)

The methylation of lipids was carried out as follows:

1. An aliquot of lipid in chloroform for each sample was evaporated to dryness under nitrogen in a screw-cap tube.
2. 250  $\mu$ l of Baran trifluoride methanol (20%) was added to the lipid in each tube and boiled in a closed container water bath for 90 minutes.
3. The tubes were then cooled and 240  $\mu$ l of benzene plus 250  $\mu$ l methanol added. This was again boiled for a further 30 minutes and then cooled.
4. 3,6 ml pentane plus 0,71 ml  $H_2O$  (5:1) was added to each tube which was then shaken and refrigerated overnight. The tubes were then centrifuged and the supernatant containing the fatty acid methyl esters and the dimethylacetates removed and evaporated to dryness. The residues were then dissolved in hexane and stored for GC analysis in amber vials at  $-13^\circ C$  in a nitrogen atmosphere.

APPENDIX 5GAS CHROMATOGRAPHY

Chromatography is a general term for separation processes in which the components of a mixture are repetitively equilibrated between two phases; normally one of these phases is fixed or stationary, and the other is mobile. This is the case with thin layer chromatography (TLC) where the stationary phase is the silica gel film placed, usually, on a glass plate. The mobile phase is the developing solvent mixture.

In gas chromatography where the mobile phase is a gas, either a liquid or a solid can be used as the stationary phase. This has led to the term "gas-liquid partition chromatography", shortened in modern literature to "Gas-chromatography" or GC.

In the GC process the stationary phase is confined to a long tube, the column, in which it exists as a thin film that is either distributed over an "inert" granular support (packed columns) or supported as a thin coating on the inner surface of the column (the so-called capillary columns). The column, which begins at the inlet of the GC and terminates at its detector, is adjusted to a suitable temperature and is continuously swept with the mobile gas phase (carrier gas), usually nitrogen. When a mixture of volatile components is introduced in the inlet, each constituent is carried toward the detector by the stream of carrier gas. The molecules of these components that are

more easily soluble in or exhibit stronger affinities for the stationary liquid phase require a longer period of time to reach the detector than do components that are less strongly oriented toward the liquid phase. Separation is thus achieved.

A compound in a column will, therefore, spend some of its time in the liquid stationary phase and the rest in the gas phase, each substance having a different equilibrium constant which is governed by the compound, the liquid phase and its affinity with the compound, and the temperature of the column. Liquid phases are classified according to their polarity: the most polar liquids are capable of forming strong hydrogen bonds, while the least polar can interact only by forming weak Van der Waal's bands (Zweig and Sherma, 1972). Compounds to be analysed can also be classified in the same way. Thus liquid phases which are similar to the components retard these components compared to liquid phases which are not similar. Thus, certain columns are coated with specific substances for the work they are designed to do. The columns designed for fatty acid methyl ester analysis are coated with polyesters (Zweig and Sherma, 1972).

Though constituents of a limited boiling point range can usually be investigated under isothermal operating conditions where the highest separation efficiencies are normally realised (Jennings, 1980), better resolution at the low end and faster elution at the higher end is obtained if temperature is progressively increased, for mixtures of wide boiling ranges. Temperature programming combines the benefit of the improved separations at lower column temperatures with the advan-

tage that the detector receives each solute as a sharp, narrow burst. At the beginning of the program, the column temperature is low; lower-boiling components separate and are carried by the gas, whilst intermediate-boiling components move very slowly up the column in the liquid phase, and the higher-boiling components stay behind.

As the temperatures increase the intermediate boiling compounds begin the chromatographic process of separation and finally the higher-boiling components undergo the partitioning process, by which time the earlier compounds have already passed through the column and reached the detector. Thus pulses of substances reach the detector giving sharp, narrow peaks of high concentration allowing better identification and quantification of the substances (Jenning, 1980).

The detector used is generally a flame ionization system.

Identification of peaks is done by injecting known substances into the column at a particular temperature programme. The relative retention time of these substances (i.e. the time for these substances to reach the detector) are recorded. The peaks observed from a mixture of substances can thus be identified from these standard peaks and quantities calculated by an integrator calculating the areas of the peaks. Thus, the percentage of each peak in a mixture can be calculated.

Good reviews on Gas Chromatography are given in Jennings (1980) and Zweig and Sherma (1972).