Thesis
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A Study of Embryonic, Larval and Postlarval Responses to Conditions of Water Hardness and Alkalinity in *Macrobrachium rosenbergii* (De Man) 1879

Thesis submitted to the University of Stirling

for the degree of Doctor of Philosophy

By

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DEDICATION

I dedicate this chapter of my life to my beloved

CHARLOTTE WELLON

CHARLOTTE

I have no words to describe why I

want to dedicate this to you, but

I think that you know it….

C

ACKNOWLEDGMENTS

FEMALE, the beautiful side of universe

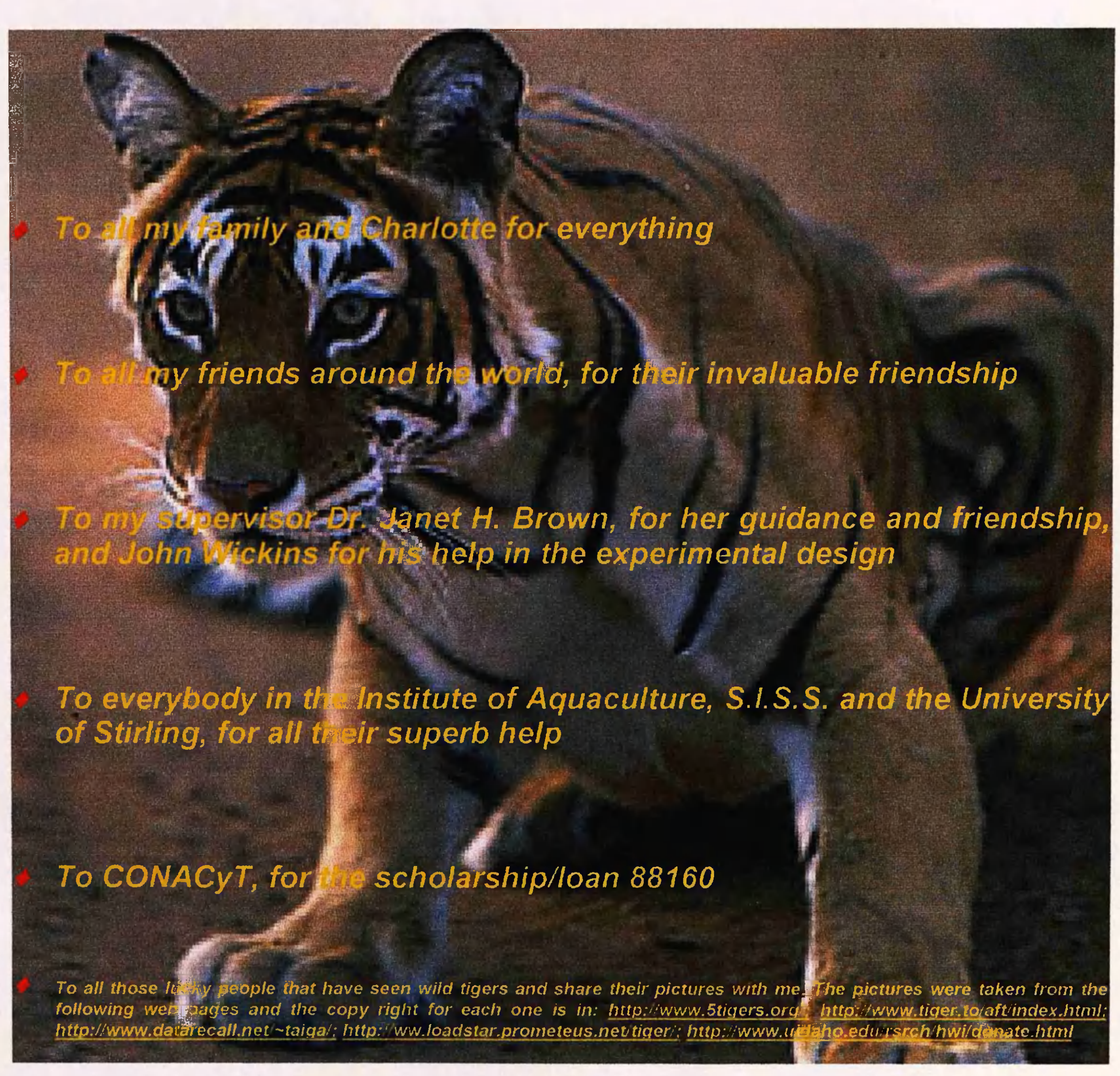
LOVE, the climax of existence

I loved you when I was looking for you,

I found you and I loved you even more,

now….

ACKNOWLEDGMENTS



DECLARATION

This thesis is a compilation of my own research work and it has not been submitted for any other qualification. All the information from other sources has been duly acknowledged.

Conrado González Vera

ABSTRACT

Macrobrachium rosenbergii (De Man) has been transferred to several sites around the world for its potential of culture and commercialisation, however, some countries like Mexico, have limitations regarding the levels of calcium carbonate in its waters. Up to now there is some controversy in the results of the effect of calcium carbonate on the development in the species, therefore, the responses of embryonic, larval and postlarval *M. rosenbergii* to different environmental conditions of water hardness and alkalinity were investigated.

This study was made in two separate stages, the first investigated the effects of alkalinity and total hardness as two separate factors affecting the performance of postlarvae, and the second investigated the possibility of adjusting the tolerance limits of the species to alkalinity, the most important of the two environmental variables.

Alkalinity reduced the growth rate and survival of postlarvae of M. rosenbergii, and at the same time increased their moult frequency. Besides, it was found that an alkalinity concentration of 250 mg L^{-1} (as $CaCO_3$) or above was lethal for postlarvae of the species. Total hardness as high as 1000 mg L^{-1} (as $CaCO_3$) however, did not have any significant effect in the growth rate or survival as long as the alkalinity was 100 mg L^{-1} (as $CaCO_3$) or lower.

Acclimatisation of the embryos of *M. rosenbergii* to alkalinity levels as high as 200 (mg L^{-1} as $CaCO_3$) did not reduce in a significant way the effect of high alkalinity in the postlarval stages of the species.

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CHAPTER 1

1. GENERAL INTRODUCTION

Wild freshwater prawns of the genus *Macrobrachium* spp. have been heavily exploited wherever they are found. *Macrobrachium rosenbergii* is the most exploited species of all, and in some areas of Asia its captures have declined (local areas of Indonesia and India), due to the increase of the human populations and the development of new catching techniques (Rabanal, 1982; Jacob and George, 1993) bringing with it an increase in price (Bian and Pang, 1982; Rise, 1999). In the decades of 1950 and 1960, the demand for this species grew very fast and methods for its culture were developed (Ling, 1969; Uno and Kwon, 1969; Fujimura and Okamoto, 1970). Nowadays, the species is successfully cultured in countries like Thailand and Taiwan, among others (FAO, 1998).

1.1. GLOBAL MARKETING OF *M. rosenbergii*

1.1.1. Demand

Prawns of the genus *Macrobrachium* are consumed as a delicacy all over the world (New, 1982; Rabanal, 1982; Singh and Srivastava, 1989). *M. rosenbergii* has a reputation for its better taste against *Penaeus* spp (Johnson, 1966; Meknavin, 1982; Nash *et al.*, 1987; New, 1995; Rise, 1999), and this characteristic gives to *M. rosenbergii* the advantage to participate in both the common shrimp market (New, 1995; New, 1996) and in a specific market for its

taste (New, 1995); for example in Veracruz (Mexico) there is a high demand for *Macrobrachium* species due to their preferred flavour, even when the supply of *Penaeus* species is quite high in the region (Reta Mendiola, 1999). The demand for this species in local markets where the organisms are produced, is as high as in the large markets (USA, Japan and France) (Meknavin, 1982; New, 1982; Taw, 1982). The USA is a big market for freshwater prawns (Liao and Smith, 1983), which imports them from several countries that have programmes of small scale aquaculture like the family backyard prawns production in Thailand for instance (Brown, 1991). Japan and France are the second and third largest markets of prawns, in which the demand for these organisms is still unsatisfied (New, 1995). There is a prediction by FAO that during the next century fish consumption will drop in Europe and North America, due to price competition with chicken and pork (Josupeit, 1999). However, crustaceans have always been a high priced product and therefore the demand may not be severely affected by this change of prices.

1.1.2. Supply

1.1.2.1. Fisheries production

Although fisheries of *M. rosenbergii* have almost doubled from 5439 to 10,083 metric tonnes per year (t $year^{-1}$) during a period of nine years from 1987 to 1996 (FAO, 1998), the supply cannot satisfy the demand for the product, even in local markets, because the fishery capacity has been

exhausted in many areas (Rabanal, 1982). The bulk of the fisheries production of *M. rosenbergii* is mainly in the South East Asia region (FAO, 1998), thus being related to its natural distribution (see section 1.3.). Indonesia has been the major fisheries producer of the species on a global scale, with 5330 t $year^{-1}$ in 1996, followed by Thailand, although the latter suffered very acute drops in its captures from 1989 to 1993 (FAO, 1998).

1.1.2.1. Aquaculture production

There has been a steady growth in the production of *M. rosenbergii,* since the completion in captivity of its life cycle. A complete review of the aquaculture production of *M. rosenbergii* was reported by New in 1995, in which he mentioned that according to FAO, in 1992 Asiatic aquaculture of *M. rosenbergii* was 92% of the total production by this industry world wide, while the other 8% was produced in the Americas. By 1997 China had taken the leading role in the aquaculture of *M. rosenbergii*, followed in descending order by Thailand, Taiwan, Ecuador, Brazil, India, Malaysia and Mexico, and the global production was amounted up to 60,995 t $year^{-1}$ (FAO, 1999).

There have been several attempts to develop the culture industry of *M. rosenbergii* in some countries of Latin America, but because of the inconsistency of the production there has not been much success, specially in countries like Mexico, Costa Rica and Colombia among others (Ruiz and Ruiz, 1987; New, 1995; Bottero, J. and Reta Mendiola J.L., personal communications,

1997 and 1999, respectively). These countries have a huge potential for the developing of the prawn industry, and they could certainly benefit from following the example of Taiwan, where freshwater prawns have received as much attention as marine shrimps (Liao and Chao, 1982).

1.2. SYSTEMATICS

As a freshwater organism *M. rosenbergii* is referred to as "prawn" (FAO, 1967, cited in: Csavas, 1988) and is taxonomically classified as shown in Table 1., according to several authors cited in: Green, 1961; Wickins, 1976a; Holthuis, 1980; Bowman and Abele, 1982.

Table 1. Taxonomy of *M. rosenbergii*.

Phylum	Arthropoda
Superclass	Crustacea (Pennant, 1977)
Class	Malacostraca (Latreille, 1806)
Subclass	Eumalacostraca (Grobben, 1892)
Superorder or Division	Eucaridea (Calman, 1904)
Order	Decapoda (Latreille, 1803)
Suborder	Natantia or Pleocyemata (Burkenroad, 1963)
Infraorder or Section or Tribe	Caridea (Dana, 1852)
Superfamily	Palaemonoidea (Rafinesque, 1815)
Family	Palaemonidae (Rafinesque, 1815)
Subfamily	Palaemoniae (Rafinesque, 1815)
Genus	*Macrobrachium* (Bate, 1868)
Species	*rosenbergii* (De Man, 1879)

This species is known by several common names around the world, such as the ones shown in Table 2.

Table 2. Common names for *M. rosenbergii*.

Common name	Location	Reference
Udang galah	Malaysia, Indonesia	Ling, 1969; Holthuis, 1980; Melatunan, S., personal communication, 1997
Crevette tropicale d'eau douce	French Territories	Griessinger *et al.*, 1991
Langostino, langostino azul, langostino gigante, langostino malayo, gamba, camaron de agua dulce	Spain and Latin America	Avendaño Morales, 1994; personal observations
Koong yai, mai koong, koong ruan, koong gamgram	Thailand	New *et al.*, 1982; Holthuis, 1980
Giant freshwater shrimp, giant freshwater prawn	USA	Holthuis, 1980
Udang satang, udang duri	Indonesia	Holthuis, 1980
Bharo chingri, chooan chingri, mota chingri, shala chingri	Bangladesh	Holthuis, 1980
Golda chingri, mocha chingri, aattu konju	India and Bangladesh	George, 1969; Holthuis, 1980
Freshwater prawn, freshwater shrimp, blue lobster, giant long legged Malaysian prawn, freshwater Malaysian prawn, Hawaiian prawn	Various	Ling and Merican, 1961; Ling, 1969; New, 1988

However, its official common names according to FAO are: giant river prawn (English), bouquet géant (French) and camarón gigante (Spanish) (Holthuis, 1980), or langostino de río (Spanish) (FAO, 1999).

1.3. GEOGRAPHICAL DISTRIBUTION

M. rosenbergii is naturally present in Malaysia (Johnson, 1967) and India, with the east of India being the most western distribution area (George, 1969). Indigenous to the Indo-West Pacific, this species is found in several countries, like Bangladesh, Myanmar, Thailand, Indonesia, Papua New Guinea, north Australian coasts, Philippines, Cambodia, Vietnam, Taiwan and south Chinese coasts (Holthuis, 1980). However, nowadays the giant freshwater prawn is found in many countries around the tropical and subtropical belts. Its wide distribution is because mankind has artificially introduced it to new places (New, 1996) in order to develop its culture, since it is the largest species of the genus, has a high resistance capacity to variations in the culture media and is highly priced.

1.4. GENERAL BIOLOGY OF THE SPECIES

1.4.1. Life cycle

Details of some aspects of the life cycle of *M. rosenbergii* have been described in detail by several authors, e.g. Ling, 1962, 1969; George, 1969; Uno and Kwon, 1969; Wickins, 1976a; New and Singholka, 1982). The four main phases of the cycle are egg (embryo), larva, postlarva, and adult which are represented in Figure 1.

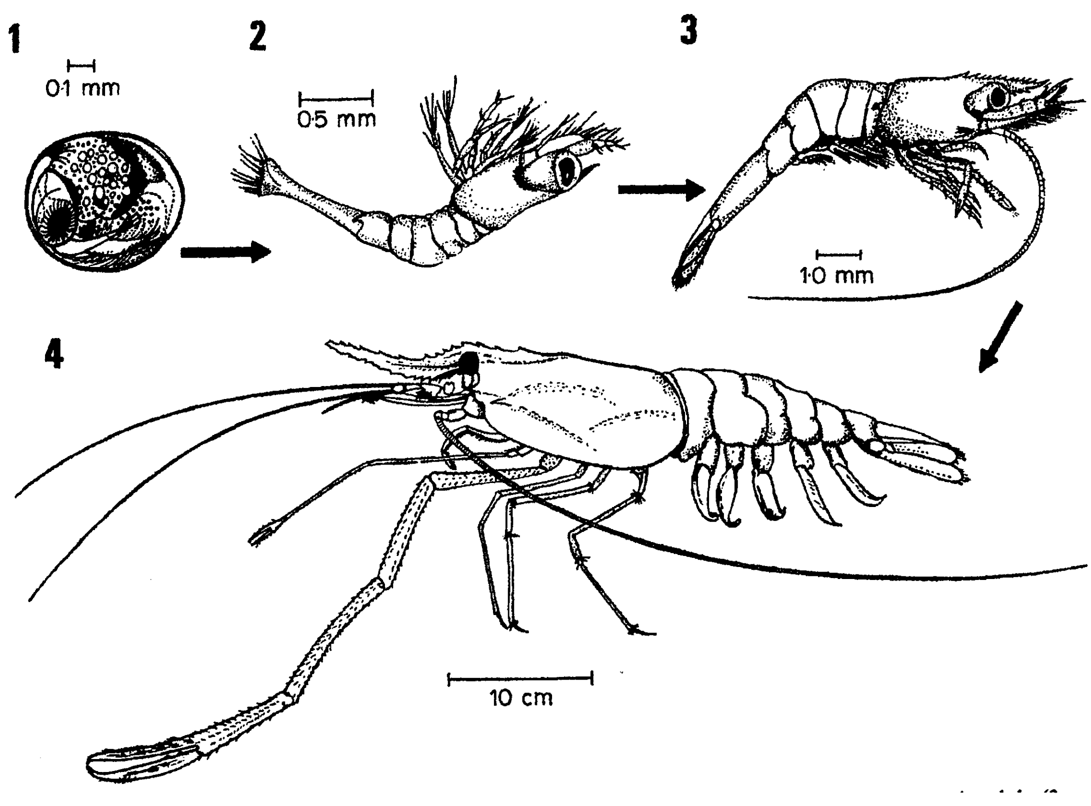


Figure 1. Life cycle of *M. rosenbergii*, taken from Forster and Wickins (1972).
* Numbers represent the different stages of development, namely egg (embryo) = 1, larva = 2, postlarva = 3 and adult = 4.

1.4.1.1. Embryos

Under laboratory conditions, the embryonic development takes at least 17 days (this study) and could extend to up to 19 days (Ling and Merican, 1961; Ling, 1969) or 20 to 21 days (John, 1957) due to environmental conditions and probably to health status and possibly genetic variations of the broodstock. Gomez Diaz (1987) found that embryos of *M. rosenbergii* hatched at 26, 17 and 16 days depending on the temperature (25, 29 and 31 °C respectively). Sexually mature adults mate in their natural environments once the female has moulted and she then carries the eggs attached to her

swimming legs (pleopods) until they hatch. Some large females can produce well over 100,000 eggs per brood (Ling, 1969; Lee and Wickins, 1992). The eggs are slightly oval in shape and they change from a dark yellow coloration, in the early stage of development, to a slate grey when they are ready to hatch (George, 1969; Ling, 1969; Uno and Kwon, 1969). During this period of time the female migrates downstream towards the sea, in order to reach brackish water environments (John, 1957; George, 1969) like estuaries or coastal lagoons.

1.4.1.2. Larvae

When the embryos are ready to hatch as free swimming larvae (stage I), they are in, or very near to, brackish water. Larvae in their first developmental stage have the capacity to tolerate freshwater for a period of 5 days maximum, after that, they will die if they are not in a brackish water environment (Johnson, 1967; Ling, 1969). Once they are in optimum salinity conditions (12 ‰, Ling, 1969), the development of the larvae goes through eleven developmental stages, and each of these stages brings a new morphological characteristic with itself. Ling in 1969 reported 8 different stages for the larval development, but eleven moults during the development of the organisms. In the same year, Uno and Kwon reported eleven stages of development and gave a detailed description of each one of them, whilst Gomez Diaz and Kasahara in 1987 increased that description to seventeen different stages. The description made by Uno and Kwon in 1969, was used in this study, since it describes each one of the moults mentioned by Ling in 1969

and each of their described stages shows a very easily identifiable morphological characteristic, which is not the case in the description made by Gomez Diaz and Kasahara (1987), for the 17 different stages they identified.

1.4.1.3. Postlarvae

Once the larvae reach stage eleven, the next step is to metamorphose into postlarvae, which resemble miniature adults. At this stage of the life cycle, the prawns change from living suspended in the water column as part of the plankton, to be crawling organisms at the bottom (Ling, 1969; Uno and Kwon, 1969). The postlarvae develop a positive rheotaxis and they start crawling against the freshwater current upstream, though sometimes they can stay in brackish water for as long as two weeks (Ling, 1969). Postlarvae grow continuously in optimal conditions and the "juvenile" term although it is not well defined, it is commonly used for the organisms that are in between the postlarva and adult stages, usually after they become of a brownish and bluish coloration (D'Abramo *et al.*, 1996). The "juvenile" term could be used for organisms that are between the postlarvae and adult stages before reaching sexual maturity.

1.4.1.4. Adults

Finally after a period of time (depending on the external conditions and the fitness of the organisms) the postlarvae reach the adult phase. They

produce their first broods after the first year (New and Singholka, 1982). When the females are sexually mature and after the "pre-mating" moult, they mate and produce eggs that are incubated while they migrate downstream, reaching salinity levels around 12 ‰ for the hatch of the larvae, to start the whole cycle again. From the larval stage and up to adulthood, *M. rosenbergii* presents differences in the growth rate, and behaviour (social hierarchy) among organisms from the same age group, this is discussed latter on in section 1.5.2.1.

1.4.2. Habitat

Due to the life cycle of this species, the habitat of *M. rosenbergii* covers fresh and brackish water and sometimes it is found in marine waters (Holthuis, 1980). As a larva, it is found in estuaries, coastal lagoons or deltas, meanwhile as a postlarva it goes from the previous environments, to rivers, canals, and streams that run towards the sea (John, 1957; George, 1969). As an adult it has been found from 0 to 200 km from the coast, in rivers, irrigation ditches, swamps, ponds, mining pools, water reservoirs, paddy fields, canals, and even in estuarine areas (Ling, 1969; New and Singholka, 1982). Although some species of prawn prefer to live in clear water, *M. rosenbergii* prefers the turbid environments (New and Singholka, 1982). *M. rosenbergii* is able to crawl in very shallow waters and even in just wet land in order to reach neighbouring waters like paddy fields for example (John, 1957; New and Singholka, 1982).

1.4.3. Feeding behaviour

M. rosenbergii is an omnivorous species and can eat raw vegetables when is in the wild and roasted bran (John, 1957), commercial pelletised feed (Weidenbach, 1982) or egg custard (Malecha, 1983a) when is in captivity. When the prawns are embryos, they use nutrients provided by the female during the ovarian maturation, which are very rich in lipids and proteins, and which last until they are in their first stage of the larval development (Ling, 1969). Once it moults into stage two, it starts feeding on small phytoplankton as well as zooplankton and, if there is a lack of food, the stronger larvae could feed on the weaker ones (Ling and Merican, 1961). From metamorphosis and up to the whole adulthood, they feed basically on almost everything they can manage to catch. Their natural diet varies according to their environment (John, 1957) and goes from aquatic insects in all their developmental stages, micro and macroalgae, nuts, grains, seeds, fruits, to fish, molluscs (including small pieces of shell) and they even practice cannibalism when they are in high densities or there is a lack of food (John, 1957; New and Singholka, 1982).

1.4.4. Physiology of moulting and exoskeleton mineralization

The calcareous exoskeleton in a decapod crustacean provides protection and support, but also imposes severe limitations, since growth is only possible by complete shedding and regeneration (moulting) of the exoskeleton.

1.4.4.1. Structure and composition of the exoskeleton

According to Richards (1951) the integument of the decapod crustacean is composed of an exoskeleton and a cellular hypodermis. The layers of the exoskeleton are described with different nomenclatures, depending on the authors, but the characteristics of each layer in the exoskeleton are unique. Travis (1960a) and Roer and Dillaman (1984) named the layers of the exoskeleton as epicuticle, exocuticle or pigmented layer, endocuticle and membranous layer (from the most external to the most internal, respectively) and hypodermis to the next internal layer. Stevenson (1985) named those layers in the same directional order, but as epicuticle, procuticle and then followed by the epidermis. The latter nomenclature is the one used nowadays and therefore it will be employed in this study. There are several detailed papers describing the cuticle composition of crustacean, e.g. Travis (1960a); Richards (1951); Roer and Dillaman (1984) and Stevenson (1985) among others, therefore, only a concise description of it will be given in this section.

Figure 2. Shows the different layers in the exoskeleton of a crustacean decapod. The epicuticle is the thinner and outer layer of the exoskeleton, which is mainly composed by protein and lipids impregnated by calcium salts. The next layer is the procuticle, which is divided into two more layers, i.e. preecdysial and postecdysial. The postecdysial procuticle then is subdivided into the principal and membranous layers. The procuticle is composed of the same elements as the ones forming the epicuticle, the difference is that it has chitin and proteins forming fibres, instead of lipids,

however, the membranous layer and membranous areas of the procuticle have no calcium salts in their composition (Travis, 1960a; Roer and Dillaman, 1984; Stevenson, 1985). The principal layer is the thickest and the most heavily calcified layer in the exoskeleton. The responsible mineral for the hardening of the exoskeleton is calcium carbonate in the form of calcite or poorly crystalline and amorphous calcium, although some other elements are present in a relatively small quantity, i.e. magnesium, strontium and phosphorus (Travis, 1963; Fieber and Lutz, 1985; Greenaway, 1985). The membranous layer is the next external layer after the epidermis, the last one has three main layers of cells, and the most external of these is a single cell thick layer, which is the responsible for the secretion of the cuticle (Richards, 1951). Other structures related to the secretion of the cuticle are the extensions of the integumentary glands, which are extended into ducts or canals penetrating the cuticle, those canals could be up to 4,000,000 per mm^2, depending on the species (Richards, 1951; Travis, 1960a; Roer and Dillaman, 1984; Stevenson, 1985).

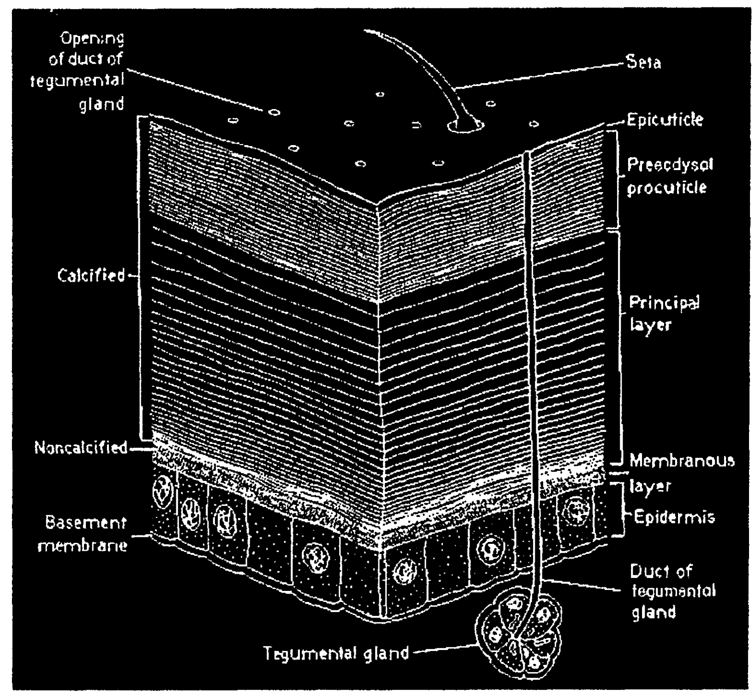


Figure 2. Crustacean cuticle (taken from Stevenson, 1985).

1.4.4.2. Moulting

Several papers describe in detail the moulting process and its metabolism for different species of crustacean e.g. Travis, 1957; Passano, 1960; Skinner, 1985. Travis (1957) describes metabolic changes in the hepatopancreas and integumental tissues of post-ecdysial *Panulirus argus* during the moulting cycle. Passano (1960) describes the moulting cycle, the control organs and the stimulus that have influence on it. Skinner (1985) describes in detail the moulting cycle in crustaceans as the processes involved in growth, which has several events, i.e. formation of gastroliths (in some species, but not in Natantia), atrophy and replacement of somatic tissue in the chelae, degradation and shedding the old exoskeleton, regeneration of missing appendages and generation of the new exoskeleton. Although moulting occurs frequently in crustaceans, in order to allow size increments, and then growth through the replacement of water by mainly muscle protein when in optimal conditions (D'Abramo *et al.*, 1996), growth does not always occur in every single moult.

Hardening and calcification of the new exoskeleton after shedding the old one (which is called exuviation or ecdysis) and its subsequent decalcification and then the formation of a new exoskeleton, occurs along the whole life cycle of the crustacean (Huner and Avault, 1977; Roer and Dillaman, 1984; Skinner, 1985). However, Passano (1960) mentions that some species of decapods reach an upper limit of size and moulting stops. The moulting process was divided into five main stages by Drach in 1939 (Travis, 1960a; Dall, 1965;

Roer and Dillaman, 1984; Skinner, 1985) and the different stages are: intermoult, premoult, ecdysis, immediate postmoult and postmoult.

Figure 3., shows the different structural developments through the moulting cycle in crustacean.

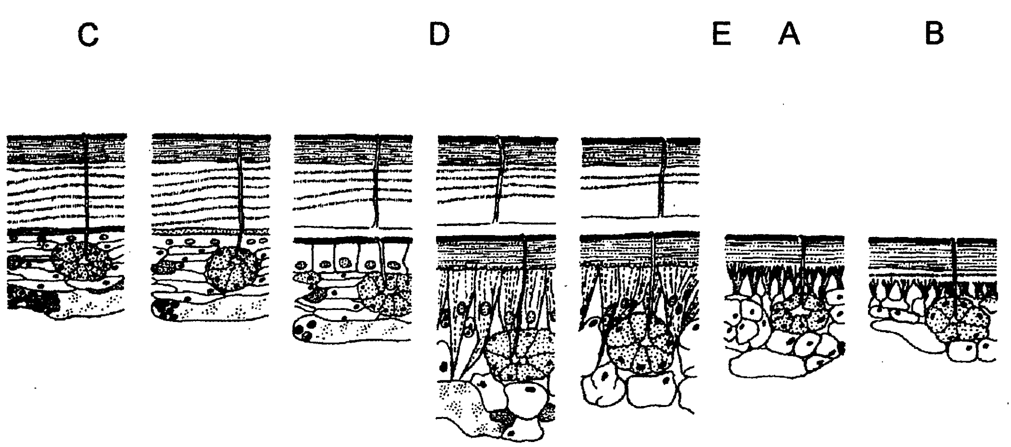


Figure 3. Moulting sequence in a crustacean exoskeleton, after Skinner (1962, cited in: Skinner, 1985).
A = Immediate postmoult; B = Postmoult; C = Intermoult; D = Premoult; E = Ecdysis.

The intermoult or stage C has been divided in four sub-stages. C_1, the first sub-stage consisting of the chemical changes in the cuticle and main tissue continues growing. The second sub-stage (C_2) being the starting of the hardening of the exoskeleton, and C_3 is when the exoskeleton is completely hardened and the membranous layer is formed. At the fourth sub-stage (C_4), membranous layer and hardening of the exoskeleton is completed and the intermoult starts with a major accumulation of organic reserves. At stage (D) or premoult, there are again four sub-stages. Apolysis occurs in sub-stage D_0 and the cuticle starts separating from the epidermis, regeneration of lost appendages starts, atrophy of somatic muscle in chelae occurs and the

hepatopancreas is activated for storage of organic reserves. The appearance of the new setae, resorption of the old exoskeleton and increase of epidermal cells occurs in D_1. In D_2 the new epicuticle is secreted along with some procuticle. In D_3 most of the old postecdysial procuticle is digested and reabsorbed, letting the organism ready for stage D_4, where the old exoskeleton is split open as absorption of water increases the size of the organism. After that, stage E or Ecdysis occurs, here the prawn loses its old exoskeleton and a rapid water uptake is carried out. During the immediate postmoult stage (A), the epidermal cells get smaller but water continues to be absorbed, the new exoskeleton is soft and the animal starts eating its old exuvium (shed exoskeleton). At postmoult (stage B), formation of the procuticle starts, the setae are retracted and the cones where the new setae will be developed are created, the chelae start to be hardened and the tissue growth begins. (Drach, 1939; Passano, 1960; Stevenson, 1985; Dall, 1965; Travis, 1960a; Skinner 1985). Stevenson (1985) mentioned about his proposed universal criteria in 1968 for the definition of the moulting cycle in crustacean, concluding that each investigator uses and defines its own descriptions according to the new group of organisms that they are observing. Therefore, although the moulting description by Drach in 1939 is a generalised one, there are some variations according to each author.

The moulting cycle of *M. rosenbergii* starts just after hatching. It can moult as often as every day in the first two larval stages, but the frequency of moulting starts to decrease as the organism grows, and it can be up to every 40 days during the adult stage (Ling and Merican, 1961; Ling, 1969). Although this shows that *M. rosenbergii* can deal with the stressful process of moulting

constantly, there are variations in the moulting frequency from one individual to another. Furthermore, added stress caused by external factors like temperature, humidity and light can alter the moulting frequency in some species of crustacean (Passano, 1960). In the study of Howe (1981) for instance, he found that the moulting frequency in a group of prawns increased until the water in the system was changed, and once the water was changed the moulting frequency decreased significantly. He also suggested that some moult inhibition factor in the new water was adding, rather than removing, a moult inducing factor within the old water, furthermore, he mentioned that moulting animals could influence a nearby animal to moult. This response of crustaceans to moult when they are stimulated by external factors has been used with advantage in the shrimp industry. Ecuadorian shrimp farmers have seen that whenever the low tides produce high temperature, the shrimps moult in synchrony, and the farmers take advantage of that in order to obtain soft shell organisms for a specialised market (Lutz, C.G. and Barcillo, personal communications, 1998). Handling the organisms or a quick change of the total hardness or alkalinity in the water can induce a premature moult in postlarvae of *M. rosenbergii* (personal observations). In 1991, Brown *et al.,* observed that the moulting rate in postlarvae of *M. rosenbergii* was low when the environmental hardness was at low levels, compared with high moulting frequency of organisms reared at high hardness. Cripps and Nakamura (1979), however, observed a similar reduction in the moulting rate of adults of *M. rosenbergii*, but in this case when the hardness was increased.

Moulting is a high energy consumption process and the newly-soft exoskeleton exposes crustaceans to a high risk of mortality, but some species of crustacean have developed some strategies to reduce vulnerability during this stage. *M. rosenbergii* for instance, tends to occupy less preferred substrates when it is about to, or just after, moult (Howe, 1981). The same author suggested that an unknown factor is released into the surrounding area by a moulting prawn in order to influence other possible cannibals (prawns) to moult as well. If that is so, then the conditions would be more safe to moult in an area where most of the conspecific competitors are in the same disadvantaged state. However, due to this vulnerable stage during the moulting phase, *M. rosenbergii,* as many other crustaceans, has the necessity to quickly harden its new exoskeleton for a better protection and mobility. Therefore, the quick hardening of the exoskeleton is of vital importance and it requires fast transport of essential ions like calcium and carbonate. Those ions which are the main inorganic components of the exoskeleton (Passano, 1960; Dall, 1965), are normally taken from the water media, although they could partially come from the food they ingest (once they start eating) as well as from their own metabolic CO_2.

1.4.4.3. Exoskeleton mineralization

There are several detailed works describing the exoskeleton mineralization process in different crustaceans, for instance, Passano (1960) and Stevenson (1985) and most of them refer to calcium and carbonates, due

to their importance in the mineralization process. Some other like Graf (1978) describe the exogenous as well as the endogenous calcium sources for that process.

The exoskeleton mineralization is done by two processes:

a) A partial demineralisation of the old carapace, which occurs prior to ecdysis, and where calcium is retained in the haemolymph and hepatopancreas (Travis, 1960a; Greenaway, 1974c, 1976, 1985; Fieber and Lutz, 1982; Sparkes and Greenaway, 1984). Gastroliths are a reserve centre in some species of crayfish, lobsters, marine and land crabs, which are not present in Natantia decapods like *M. rosenbergii* (Travis, 1960a,b; Mizuhira and Ueno, 1983; Sardá *et al.*, 1989). These three areas of accumulations of calcium are reused for the hardening of the new exoskeleton, though some of the calcium is lost together with the old exoskeleton and the reserves are not large enough to replace these losses (Lahti, 1988).

b) An inward transport of calcium and bicarbonate from the external media, and an outward movement of hydrogen ions, mainly carried out through the gills and to a lesser extent through the carapace epithelium (Dall, 1965; Roer, 1980; Roer and Dillaman, 1984; Cameron, 1985a,b; Cameron and Wood, 1985; Neufeld and Cameron, 1993).

Travis (1960b) described the changes in the gastroliths of the crayfish *Orconectes* (*Cambarus*) *virilis,* where he found that, prior to ecdysis, during the premoult stage "D", there was an increasing accumulation of calcium in the reserve sites and, once the moult was shed, the synthesis of the calcium started to build up in the new exoskeleton and stopped at the intermoult stage. McWhinnie (1962) mentioned that calcium ions resorbed from the old exoskeleton are bound to the haemolymph proteins to be conserved during the premoult stage in some freshwater crustaceans, whilst Chen *et al.*, (1974) identified the mitochondria in the hepatopancreas of *Callinectes sapidus* as a major storage space for calcium prior to ecdysis. Sardá *et al.,* (1989) presented a table comparing some aspects characterising the calcium metabolism in high and less calcified species (Table 3.), *Homarus gammarus* and *M. rosenbergii* respectively.

Table 3. Calcium metabolism in high and less calcified crustaceans after Sardá *et al.*, (1989).

Heavily calcified crustaceans	Lightly calcified crustaceans
Percentage of calcium in carapace between 10 and 15% in stage C	Percentage of calcium in carapace between 10 and 20% in stage C
Long intermoult period	Short intermoult period
Gastroliths present	Gastroliths absent
Hepatopancreas activity in calcium metabolism slight	Hepatopancreas activity in calcium metabolism intense in stages D_2-A
Calcium levels in hemolymph constant	Calcium levels in hemolymph constant
Penetration of calcium via gills; alternatively, through digestive tract	Low levels of calcium penetration through gastric epithelium
Reabsorption rapid from D_2	Reabsorption slow from D_0
High synchronism between growth and reproduction	Low synchronism between growth and reproduction
Chiefly Reptantia	Chiefly Natantia

Simkiss (1976) made a short explanatory review of calcification and mineralization processes. He describes a general model for calcium transport, in which calcium and carbonate are actively moved into the fluid adjacent to the sites of mineralization, where they are accumulated until they exceed the saturation level so then the mineral deposition occurs. Greenaway (1974a) presented a model, regarding the calcium uptake in the mineralization of the new exoskeleton for the freshwater crayfish *Austropotamobius pallipes*, which is similar to the one described by Cameron (1985b) for the marine crab *C. sapidus* (Figure 4.).

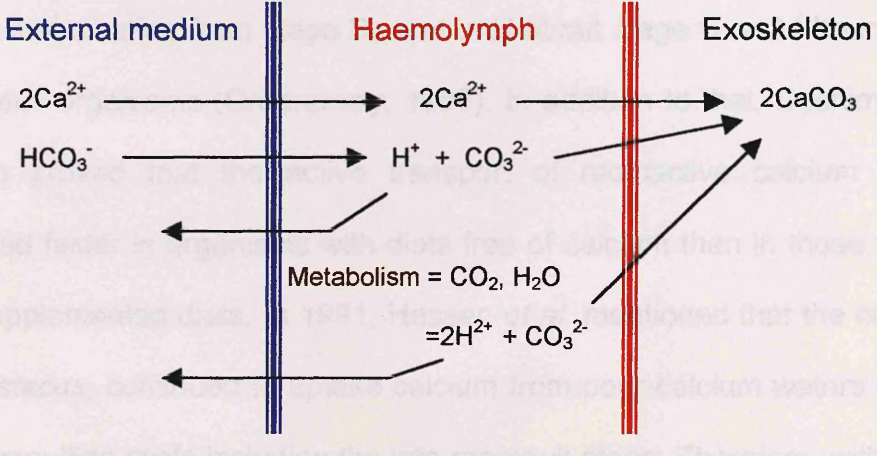


Figure 4. Calcium carbonate model for exoskeleton mineralization in crustacean, adapted from Greenaway (1974a) and Cameron (1985b).

That same model is also applicable to the findings of Malley and Tinker (1979) with the freshwater crayfish *O. virilis* and those of Fieber and Lutz (1982) with the prawn *M. rosenbergii*, where both studies showed the necessity of calcium uptake from external sources, essentially the aquatic environmental

calcium, to achieve the mineralization of the exoskeleton. Since early reports like the one presented by Numanoi in 1934, describe the changes of calcium in the exoskeleton; in his study, Numanoi found that the isopod *Ligia exotica* loses 4% of its carapace calcium content with each moult. Fieber and Lutz (1982) suggest that 57% of the calcium used in the hardening of the new exoskeleton of *M. rosenbergii* is from the reserves accumulated in the premoult stage, whilst Greenaway (1974b) estimated that only 34% of the old exoskeleton calcium content was stored by the freshwater crayfish *A. pallipes.* Although these two reports suggest that 43 and 66% (respectively) of the calcium must originate from external sources, the two options that the organisms have to acquire their required calcium are the water dissolved calcium and the feed calcium content. However, the latter one is of minimal help until late postmoult, since the organisms stops eating from stage D_3 and until about stage B or 24 hours after moult in adult organisms (Greenaway, 1985). In addition to that, Deshimaru *et al.*, (1978) proved that the active transport of radioactive calcium (^{45}Ca) accumulated faster in organisms with diets free of calcium than in those fed on calcium supplemented diets. In 1991, Hessen *et al.* mentioned that the crayfish *Astacus astacus*, continued to uptake calcium from poor calcium waters during the whole moulting cycle including the late premoult stage. Therefore, with large losses of calcium during exuviation and a constant uptake from the external media, it is evident that the presence of dissolved environmental calcium is essential apart from the possible calcium content of the food once the organisms start eating again.

As calcium is in the form of carbonate in the exoskeleton, the alkalinity concentration of the external media is directly related to the mineralization process. Wheatly and Gannon (1993) demonstrated that in *Procambarus clarkii*, calcium uptake is partially dependent upon bicarbonate ions during the postmoult stage. Greenaway (1974a) found that there exists a reduction in the calcium uptake of about 60% when no bicarbonate was supplied in the medium to the freshwater crayfish *A. pallipes.* Malley (1980) found, however, that there was no inhibition of calcium uptake by exposure to low alkaline medium (HCO_3^-) in the freshwater crayfish *O. virilis*. It seems that when the environmental alkalinity is low, another source of carbonate is used. Robertson (1941) suggests that the carbonate radical is derived from metabolic carbon dioxide. In agreement with this, Greenaway (1974a) mentioned that if *A. pallipes* continues taking up 40% of its normal required level of calcium in the absence of external bicarbonate then, an alternative source of bicarbonate is indicated, and he suggests that the source could be from metabolic carbon dioxide (CO_2) and water (H_2O). However, that is an extreme condition, which highlights the necessity of the organism to have immediate access to good sources of calcium carbonate, just after moulting, in order to be able to quickly harden its new exoskeleton and regain mobility and therefore protection against predation at that critical time of the moulting cycle (Fieber and Lutz, 1982; Griessinger *et al.,* 1991).

Sardá and Cross (1984) found that along with calcium, magnesium content in *Nephrops norvegicus* decreases from stages D to E and increases from stages A to C in the moult cycle. Huner *et al.*, (1979) mentioned that

Penaeus californiensis hardens its rostrum and carapace first and afterwards the abdominal parts, presumably doing so to protect, as fast as possible, the vital cephalic-thoracic cavity where the most important organs are located. However, they mentioned that not only calcium, but magnesium as well, reached relatively constant levels in the rostrum and carapace (1.3 and 0.9% respectively) within 24 hours after moulting. Although magnesium is the second most abundant mineral in the exoskeleton, Fieber and Lutz (1985) found that the changes of this ion occurring in the extracellular fluid, exoskeleton and hepatopancreas during the moulting process are independent of any function that it could have during the hardening of the exoskeleton. Romero (1990) mentioned that magnesium is a metabolically active agent that can interfere in the process of some enzymatic reactions carried on in the muscle and hepatopancreas and that is why *P. brasiliensis* always keeps it at low levels. However, Latif (1992) found that a concentration of 1:1 in a calcium : magnesium ratio is desirable for a better growth of juveniles of *M. rosenbergii*, and therefore, that ratio was used for the experimental purposes of this study.

All this shows that environmental calcium and magnesium hardness, as well as carbonate alkalinity, play an important role in the moulting and exoskeleton mineralization processes of *M. rosenbergii* as well as for many other crustaceans. Therefore, water quality and the site selection, considering total hardness and alkalinity, are important criteria for the culture of crustacean and in particular for *M. rosenbergii*.

1.5. CULTURE OF *M. rosenbergii*

While a major aim of the aquaculture industry is to produce good quality food, aquaculture can also produce high value products mainly destined for export markets and *M. rosenbergii* tend to fill in this latter category.

Pioneering experiments by Sidthimunka around 1950 in Thailand, followed by Ling in 1962 in Malaysia, demonstrated the necessity of brackish water during the larval development of *M. rosenbergii* (New *et al.,* 1982). Ling in 1969 described the whole life cycle of the species (in captivity) after he was able to produce metamorphosed postlarvae in his experiments. After that important step in the culture technology of *M. rosenbergii*, Fujimura and Okamoto (1970) developed a methodology to produce mass culture of the species and, nowadays, many improvements have been achieved in the techniques for the production methods. These production methods are well established, and therefore, make it relatively easy to culture and to keep this species in captivity (Wickins, 1976a; New, 1982, 1995, 1996; Malecha, 1982a,b; New and Singholka, 1982; Griessinger *et al.,* 1991; Lee and Wickins, 1992).

M. rosenbergii has a wide tolerance limit to different environmental factors as shown in Table 4. Furthermore, *M. rosenbergii* can reach sizes that are considered desirable in the food-fish market (D'Abramo *et al.*, 1996) in short periods of time (4 to 8 months) with maximum total length of 320 mm and 250 mm for male and female respectively (Holthuis, 1980). These are important qualities for a cultured species in the aquaculture business.

The prawn (*Macrobrachium* spp.) industry presents some disadvantages when compared with the shrimp industry (*Penaeus* spp.) mainly because of the faster growth rate and less aggressive behaviour of the latter (Forster and Beard, 1974). However, although the shrimp industry was rapidly expanding during the last couple of decades (Briggs, 1994), statistical analyses from FAO (Josupeit, 1999) revealed that that is no longer the case. While this was partly due to the global economical crisis of 1997, particularly in Japan, which is one of the two largest markets around the world for shrimps and prawns (Josupeit, 1999), a major reason for the lack of continuing development is because of diseases and environmental problems caused by the intensive way of its culture.

In this respect, the prawn culture has the advantage in that it appears to have high resistance to diseases and its low culture density keeps better environmental conditions. Besides, its high price due to its high demand in the markets (New, 1995), make it a very competitive industry to be developed in countries like Mexico for instance, which has optimum climatic conditions for its culture in the south of its territory and is next to the large market for shrimps and prawns of the USA.

Table 4. Tolerance limits of *M. rosenbergii*, to different environmental parameters according to several authors, adapted from Gonzalez Vera (1995).

Parameter	Unit	Level			Author
		min.	Optimum	max.	
Temperature	°C.		26 – 31*		Lee and Wickins, 1992;
		19	28 – 30	35	Griessinger *et al.*, 1991;
			24 – 30		New and Singholka, 1982;
		24		31	Sandifer *et al.*, 1982;
		15.5	24 – 31	37	El-Gamal, 1982;
			27*		Hanson and Goodwin, 1977;
		24	28 – 30	32	Sandifer *et al.*, 1977.
Dissolved Oxygen	$mg\ L^{-1}$	1	6 – 8		Griessinger *et al.*, 1991;
			> 3		Verreth *et al.*, 1987;
		2.25	6 – 8		Malecha, 1983b;
			6.6 – 8.8		Sandifer *et al.*, 1982.
PH			7 – 8.5		Lee and Wickins, 1992;
			6 – 8**	9**	Griessinger *et al.*, 1991;
		4	6 – 8.5	10.5	Griessinger *et al.*, 1991;
				< 9**	Strauss *et al.*, 1991;
				9.5	Hudon *et al.*, 1987;
				9.5	Hummel *et al.*, 1987;
			7.5 – 8.2		Sandifer *et al.*, 1982.
Salinity	ppt		12 – 18*	25*	Griessinger *et al.*, 1991;
				10***	Griessinger *et al.*, 1991;
			12*		New and Singholka, 1982;
			0		El-Gamal, 1982;
		8	12 – 16	17	Sandifer *et al.*, 1977.
Total ammonia	$mg\ L^{-1}$		0	< 1**	Strauss *et al.*, 1991;
			0	17.2 (at pH=7)	Wickins, 1976a;
			0	1.8 (at pH=8)	Wickins, 1976a.
Hardness	$mg\ L^{-1}$ as $CaCO_3$		< 53		Brown *et al.*, 1991;
			40 – 120	300	Griessinger *et al.*, 1991;
			20 – 200		Vasquez, 1989;
			< 40		Zhou, 1987;
		40	< 100	150	New and Singholka, 1982;
				1060	Bartlett and Enkerlin, 1983;
			70 – 100		Sandifer *et al.*, 1982;
			65 – 200		Wickins, 1982;
				65	Cripps and Nakamura, 1979.
Alkalinity	$mg\ L^{-1}$ as $CaCO_3$		60 – 70		Sandifer *et al.*, 1982.

Levels are for adults, unless otherwise indicated (* = Levels for larvae, ** = Levels for postlarvae, *** = Levels for juveniles).

1.5.1. Use of water

Freshwater is one of the most precious natural resources, and therefore it must to be very well utilised in any human activity. The grow-out

phase of the culture of *M. rosenbergii* is carried out in freshwater systems, and since freshwater is scarce in many countries, the culture of this species should take advantage of any suitable source of freshwater. Aquaculture of this freshwater species could be integrated with those other industries already using the existent water resources. For example the discharged heated water used in thermoelectric or power stations could be used in the culture of tropical freshwater prawns (Wickins, 1976a). Haylor in 1994 suggested that aquaculture could use the water employed in the irrigation systems, either before or after it has been used. In many areas of tropical and subtropical countries, which have suitable climatic conditions for the culture of *M. rosenbergii,* there are sources of freshwater that are not being used for any other activity and they could be used for prawn production, for example the large rivers, lakes and wetlands of Mexico (Flores Nava, 1990; Reta Mendiola, 1999). Interestingly, and as a good indication for the culture of freshwater prawns in countries without plenty of freshwater resources, Tunsutapanich *et al.*, (1982) found that *M. rosenbergii* can live and produce over 1500 kg hectare^{-1} (in 6 to 8 months) in a well managed polyculture, without changing the water except for the evaporative water losses, which were replaced in their experiments.

1.5.2. Culture constraints

Despite its taste advantage and its relative ease of culture as described, *M. rosenbergii* has been rapidly overtaken in culture volume by penaeids. These could be due to a number of constraints, biological,

geographical and in terms of water chemistry that restricts its culture potential. Lacroix *et al.*, (1994) mentioned that, in fact, research is the success provider for farmers and investors, and in fact, some of these problems are being solved by several authors through more than 20 years of research.

1.5.2.1. Biological factors

1.5.2.1.1. Heterogeneous growth

M. rosenbergii does not grow uniformly; in a newly produced population from the same broodstock, it can be seen that from early stages of development, the growth rate varies from one individual to another. For example Ling (1969), mentioned that the growth rate of the larvae of *M. rosenbergii* is fairly stable up to the first three developmental stages, but then becomes irregular. However, during this study, it was noticed that after the first day from hatching, some larvae had faster development and they reached the second developmental stage earlier than the rest. In accordance with this, Hartnoll and Dalley (1981) found that the first five larval instars of *Palaemon elegans* had the largest variability. After metamorphosis, postlarvae of *M. rosenbergii* have a homogenous growth distribution curve, but as the time passes the growth curve acquires a positive skewed shape, where the majority of the sizes fall into the lower range (Cohen *et al.*, 1981; Samples and Leung, 1985). If the population density of *M. rosenbergii* is increased, the mean weight of the organisms is negatively affected (Cohen *et al.*, 1981; Wohlfarth *et al.*,

1985; Karplus *et al.*, 1986). However, Ra'anan and Cohen (1984a, 1985) found that the degree of variability in the growth of individual postlarvae is greater when kept in a group rather than individually. Furthermore, Sandifer and Smith (1975) indicated that that degree of variability in the individual growth rate of *M. rosenbergii* was unaffected by increasing density. Brown (1991) said that these differences in growth are a strong social phenomenon. Ra'anan and Cohen (1984b) found that within two weeks after metamorphosis, a size hierarchy is formed and the large "jumpers" (already "juveniles") have a higher growth rate than those with a low growth rate called "laggards" or growth-repressed prawns, and up to fifteen times larger than the mode (Sandifer and Smith, 1975; Cohen *et al.*, 1981; Ra'anan and Cohen, 1984a). In fact, there were found three different groups of postlarval size in the populations used during this thesis after 45 days from metamorphosis. This hierarchical behaviour continues and becomes more intensive when the organisms are adults. Although Cohen *et al.*, (1981) did not find any morphological variation for postlarvae and juveniles *of M. rosenbergii*, three different morphotypes have been identified in male adult populations, differentiated by developing a physical coloration ranking (claws colour), growth rates and ratio between claw length and body size (Cohen *et al.*, 1981; Ra'anan and Cohen, 1984a,b). The coloration ranking is dominated by the blue claws, followed by the strong orange claws and then by the weak orange claws (the latter are usually < 10 g). Cohen *et al.*, (1981) found a relatively stable proportion of ranking hierarchies within a population, being 5 to10% for blue clawed males, 15 to 25% for orange ones and 20 to 25% for the small males, however, Karplus *et al.*, (1986) found that these proportions change with variations in the population size. This individual hierarchy within a

population changes with time, as the weak ones become the dominant ones, commonly after the dominant ones moult at the end of their reproductive period, commit cheliped autotomy or after these are dead (Schmalbach *et al.,* 1984; D'Abramo *et al.*, 1996).

This characteristic of heterogeneous growth in *M. rosenbergii* has been a constraint for its culture due to aggression and dominance by big animals over the small ones (Forster and Beard, 1974; Ra'anan and Cohen, 1984a,b). Thus being similar to the behaviour in lobster populations (Atema and Cobb, 1980). They said that, of the many factors that influence the agonistic encounters between lobsters, size is by far the most important, involving body and claw size. However, in order to reduce the size variation in the grow-out phase, juveniles of approximately the same size are stocked into the grow-out ponds instead of postlarvae (Ra'anan and Cohen, 1983), creating with this another phase of the production, namely the "nursery phase". Here, the postlarvae (1 to 15 days old) are reared at high densities (50 postlarvae m^{-2}) and then stocked into the grow-out ponds at larger sizes (3 g) after two months (New, 1996). This technique improves the production, since the organisms stocked into the ponds are bigger and they cope more easily with variations in the environment, like temperature (Sandifer *et al.,* 1983; New, 1995). Furthermore, it allows harvesting of marketable size organisms in shorter time, where the climatic conditions do not permit a continuous production all year round (Cohen *et al.,* 1983). Partial harvest is practised as another strategy used for the reduction of variability of marketable sizes (Cohen *et al.,* 1983; Brown, 1991; New, 1995). With this technique, the large animals are harvested

periodically and that gives small prawns the opportunity to develop a faster growth rate in the absence of the dominant ones. Furthermore this technique, in combination with the addition of shelters to the pond, allows a constant production of marketable size prawns (Cohen *et al.*, 1983), as long as the climatic conditions are favourable.

1.5.2.1.2. Territorialism and cannibalism (effects on the stocking density)

The production of *M. rosenbergii* is mainly practised in extensive and semi-intensive systems, due to the high territorialism and cannibalism of the species. Therefore, the stocking densities in the grow-out phase during production are usually less than 20 organisms m^{-2} (New and Singholka, 1982; New, 1996), being optimal at 2 per m^2 in extensive systems (Wohlfarth *et al.*, 1985; Karplus *et al.*, 1986). Hulata *et al.*, (1990) found that there were not significant differences in the mean harvest weight and income, when the prawns were stocked at two different sizes (0.25 and 0.5 g, provided the stocking density was 2 m^{-2}. Limpadanai (1980) suggested that natural population densities, in small reservoirs, adjust to no more than five prawns per m^2. Negative effects in survival and growth rate increase in parallel with the stocking density (Wohlfarth *et al.*, 1985). However, the use of shelters increases surface area within the pond, raising the possibility of increasing the initial stocking density in more intensive production systems, provided a good management of the pond is applied (Cohen *et al.*, 1983). Furthermore, an increase of the

average weight of the prawns is achieved with the introduction of substrata or shelters and also gives protection for the moulting organisms which are the most vulnerable to be cannibalised (Segal and Roe, 1975; Eble, 1979), and at the same time increasing the survival (Cohen *et al.*, 1983; Ra'anan *et al.,* 1984). In 1982, Sandifer *et al.*, experimented with high stocking densities (32 prawns m^{-2}) finding that animals of an average size of 16.2 g could be harvested in 138 days, provided the ponds were well managed as well as having the addition of substrata. Although the lack of super-intensification is considered to be a problem in the expansion of production, it has the advantage of keeping low pollution in the pond environment and the surrounding waters. That helps the development of the benthos in the ponds, which is used as a source of proteins by the prawns (Sarver *et al.*, 1982; Funge-Smith, 1991; Maclean *et al.*, 1994), therefore, increasing the opportunity to use artificial diets with less percentage of proteins and at the same time a reduction of the operational costs.

1.5.2.2. Geographical factors

Tropial countries with restricted access to the coast, and therefore small possibilities to develop the shrimp industry, but which have large inland freshwater resources could develop the culture of *M. rosenbergii.* Even those countries with large coastal areas, but with large freshwater resources and optimal climatic conditions could develop this industry at the same time as the shrimp industry develops. The problem here is that although *M. rosenbergii* is a freshwater species, it necessitates brackish water in the larval phase of its

development, and therefore hatcheries located inland have the problem of sea water supply. Qureshi *et al.*, (1993) experimented with artificial sea water in hatcheries of *M. malcolmsonii* in India, finding that the prawn was able to develop through the whole larval period as in normal sea water conditions. However, they did not mention the economics involved in the use of artificial sea water compared with natural sea water in recirculation systems or the transportation of sea salt to the inland hatcheries. In any case, transportation of sea salt is cheaper than sea water itself, and by using recirculation systems the costs are reduced (Wickins, 1976a; Singholka and Sukapunt, 1982). As a regional project helping communities in both the coast and inland, the hatcheries could be established by the coast and then the postlarvae or juveniles sold to the inland farmers as an independent business.

1.5.2.3. Water quality

Although successful aquaculture of *M. rosenbergii* has been managed in some countries, the potential for this industry has not being fully achieved yet and one of the main constraints for its expansion is the water quality.

According to Table 4., in section 1.5., *M. rosenbergii* has a wide range of tolerance to most of the critical parameters in aquaculture. However, the optimum levels of combinations of both alkalinity and total hardness factors for the species are yet to be fully identified.

1.5.2.3.1. Hardness and alkalinity.

Total hardness and alkalinity are two very important aspects of water quality in the culture of *M. rosenbergii*, since they represent the concentrations of cations (calcium-magnesium) and anions (carbonates) in the water, which are necessary for the mineralization of the exoskeleton. Although hardness and alkalinity are frequently taken as similar equivalents of calcium carbonate (WQA, 1998), they can exist in very different concentrations when comparing one to each other in the same source of water. Alkalinity exists mainly as carbonates and bicarbonates, whilst hardness is mainly due to calcium and magnesium (Boyd, 1990; Boyd and Tucker, 1992; and Wurts, 1993), depending on their geological origin (Boyd, 1979). According to Wurts (1993) alkalinity is a measure of "the amount of acid (hydrogen ion) water can absorb (buffer) before achieving a designated pH" or the total quantity of base (hydrogen ion acceptor) present, and hardness is the measure of the quantity of divalent ions in the water. There are also variations in the composition of the total hardness and alkalinity themselves. Apart from calcium and magnesium, other ions such as sodium and potassium are associated with carbonates, bicarbonates, hydroxides and phosphates, so they contribute to the increase of alkalinity. Furthermore, hardness could be caused by salts of iron or aluminium in the form of carbonates, sulphates or chlorides, instead of calcium or magnesium carbonates (Boyd and Tucker, 1992; Wynne, 1993). However, the most frequently used materials in the liming of commercial aquacultural ponds are calcite and dolomite (calcium carbonates and calcium or magnesium carbonates respectively), which could be in the forms of limestone, burnt lime or

hydrated burnt lime (Boyd and Masuda, 1994). Table 5., shows some variations of the alkalinity and hardness, reported for different water sources.

As mentioned earlier (sections 1.4.4.1., 1.4.4.2. and 1.4.4.3.), the calcium, magnesium and carbonate concentrations in the external media are of primary importance for the physiological processes of the moulting cycle, as well as for the exoskeleton mineralization in most crustacean species, including *M. rosenbergii.*

Therefore, the chemical composition of the different water bodies shown in Table 5., and their variations, demonstrate that site selection is of paramount importance for the culture of crustaceans, depending on their requirements for alkalinity and total hardness. There is some controversy about the requirements of hardness and alkalinity by *M. rosenbergii*, and that is discussed later on in section 3.1.

Table 5. Total hardness and alkalinity of some water sources, according to different authors.

Country	Water source	Hardness mg L^{-1} as $CaCO_3$	Alkalinity mg L^{-1} as $CaCO_3$	Reference
Bangladesh	Pond	20 - 1000	25 - 100	Latif, 1992
Himalayan, Bhutan	Reservoir		7.9 - 83.3	Dhendup and Boyd, 1994
Bulacan, Philippines	Groundwater		0 - 361.1	Binnie and Partners, 1986
Canada	Lake		0 - 4.5	France, 1990
Capir, Philippines	Groundwater		139.1 - 514.8	Binnie and Partners, 1986
Cebu, Philippines	Groundwater		417.2 - 734.4	Binnie and Partners, 1986
Connecticut, USA	Lake		0 - 120.5	Canavan and Siver, 1994
East France	Lake	340 - 716	107 - 237.5	Vein *et al.*, 1990
Florida, USA	Lake	2 – 730	0 - 204	Canfield and Hoyer, 1988
Guri, Venezuela	Reservoir		0.0135 - 12.15	Weibezahn, 1994
Hawaii, USA	Pond		34.3 - 76.1	Iwai, 1977
India	Pond	18 - 197	40 - 173	Jana and Kundu, 1993
India	Lake		68 - 192	Ayyappan *et al.*, 1990
Jamaica	Lake		149 - 239.5	Street-Perrot *et al.*, 1993
Java, Indonesia	Groundwater	144 - 317		MacDonald and Partners/Binnie and Partners/MTS. 1982
Lugo, Spain	River	25.5 - 28.2		Camargo, 1994
Madhya pradesh, India	River	53.7 - 286	80 - 268	Sharma *et al.*, 1993
Malaysia	Reservoir		<30	Brown *et al.*, 1991
Manila, Philippines	Groundwater		152.5 - 612.4	Binnie and Partners, 1986
Mexico	Pond	71.6 - 107.4		Muñoz Cordova and Garduño Lugo, 1993
Michigan, USA	River	25 - 207	14 - 180	Stelzer and Burton, 1993
Nuevo Leon, Mexico	Ground water	940 - 1060	58 - 86	Bartlett and Enkerlin, 1983
Morelos, Mexico	Lake	1177 - 1951	157 - 255	Hernandez Becerril and Tapia Pena, 1987
Nigeria	Lake		9.8 - 17	Khan and Agugo, 1990
Northern, Greece	River	155 - 276	220 - 314	Samanidou and Papadoyannis, 1992
Northern Ireland	Reservoir	0 - 125	0 - 100	Jordan and Enlander, 1990
Ontario, Canada	Lake		0.08 - 66.4	Rybak *et al.*, 1991
Panpanga, Philippines	Groundwater		111 - 389.2	Binnie and Partners, 1986
Papua, New Guinea	Lake		12.5 - 220	Vyverman, 1994
Pearl river, China	River		30 - 129	Yunhui *et al.*, 1991
Puerto Rico, USA	River	71		Troester and White, 1991
Sao Paulo, Brazil	Reservoir		2.2 - 42.75	Gianesella Galvao and Arcifia, 1988
Sorsogon, Philippines	Groundwater		97.6 - 829.6	Binnie and Partners, 1986
Southern, Nigeria	River		2.3 - 270	Ogunkoya and Adejuwon, 1990
Sweden	Lake		0 - 18.5	Hakanson *et al.*, 1990
Texas, USA	Ground water		39 - 134	Ground and Groeger, 1994
Yucatan, Mexico	Reservoir	198 - 998	130 - 840	Flores Nava, 1990

1.5.3. Adaptability of the tolerance limits to different environmental parameters

Due to the differences in the physicochemical characteristics among different water bodies, some attempts have been made to try to adapt different aquatic organisms to new environmental conditions. Korwin-Kossakowsky (1991) experimented with the adaptation of the common carp *Cyprinus carpio* to different alkalinity concentrations in the culture media, finding that the development of the early stages of the carps reared at pH 10.3 was similar to the control group (pH = 7.8 to 8.2), but the growth rate and survival were reduced. *M. rosenbergii* has been the subject of studies concerning adaptability to different environmental parameters, for example Gomez Diaz (1987) experimented with different levels of temperature to see if the larvae of this species could change the range in which it can grow satisfactorily. He found that the tolerance limits to temperature were increased when the larvae were acclimatised to lower temperatures from a range between 25 and 31 °C. In the same study, Gomez Diaz mentioned that adaptation to variations in the media is decreased in crustacean as these organisms grow older. Latif (1992), however, found that juvenile *M. rosenbergii* were more resistant than postlarvae to stress tests using environmental calcium carbonate. Therefore, postlarvae of *M. rosenbergii* were chosen for the first phase of this thesis, in order to test the stress caused by environmental variations of total hardness and alkalinity. During the second part of this thesis embryos of *M. rosenbergii* were used for the adaptability test (see chapter 4) investigating the potential of *M. rosenbergii*

to acclimatise to different levels of total hardness and alkalinity in the rearing water media.

1.6. AIMS OF THIS RESEARCH

It has been described how hardness and alkalinity are involved in the physiology of *M. rosenbergii*. Therefore, a good understanding of the requirements of those two parameters, used by the prawns, is necessary for the site selection of its culture according to the chemical characteristics of the water supply. The findings of this study are intended to help indirectly to the expansion of the industry in different countries with optimal climatic conditions, but with different water quality. The research was divided in two main parts, with particular objectives in each one:

1) To investigate the effects of environmental alkalinity and total hardness in the development of postlarvae of *M. rosenbergii*, and to identify the maximum tolerance limits as well as the optimum range of levels of both factors, individually and as a synergistic effect for the prawns.

✓ To investigate the maximum tolerance limit of postlarvae of *M. rosenbergii* to alkalinity and in particular to carbonates in the culture media.

- ✓ To investigate the maximum tolerance limit of postlarvae of *M. rosenbergii* to total hardness in the culture media.

- ✓ To determine an optimal range of levels of alkalinity and total hardness in the culture media for postlarvae of *M. rosenbergii.*

- ✓ To investigate the synergistic effect of both factors alkalinity and total hardness in the performance of the postlarvae of *M. rosenbergii.*

2) To evaluate the effects of the acclimatisation of *M. rosenbergii* as embryo, larvae and postlarvae to different levels of alkalinity and total hardness.

- ✓ To find out the adaptation capacity of the embryos of *M. rosenbergii* to low, medium and high levels of alkalinity and a total hardness of 100 mg L^{-1} (as $CaCO_3$) in the culture media.

- ✓ To investigate the performance of larvae of *M. rosenbergii* when hatched from embryos incubated at different levels of alkalinity and a total hardness concentration of 100 mg L^{-1} as $CaCO_3$.

- ✓ To determine if postlarvae of *M. rosenbergii* can increase their tolerance limits to high alkalinity levels, after being metamorphosed from larvae hatched from embryos incubated at different alkalinity levels.

Whilst the first part of the study was intended to identify the natural tolerance limits to alkalinity and total hardness, the second one was intended to alter them, in order to evaluate the suitability of alkaline waters like the ones in the South East part of Mexico, which have excellent climatic conditions but elevated alkalinity.

CHAPTER 2

2. GENERAL METHODOLOGY

2.1. EXPERIMENTAL AREA AND FACILITIES

Two main production areas were used during this study, namely the Tropical Prawn Unit and the Tropical Aquarium Unit in the Institute of Aquaculture at the University of Stirling.

2.1.1. Tropical Prawn Unit

All the experiments were conducted in the Tropical Prawn Unit. This unit is equipped with several systems to maintain different populations of fresh, brackish and marine crustaceans. It has an air blower (Single phase D80, Electromotors), with a main distribution line, from which all the different systems are supplied.

2.1.1.1. Sea water storage system

The Tropical Prawn Unit has an external sea water reservoir (Fastank ® 10,000 L capacity) made of flexible plastic and covered to prevent contamination, phytoplankton blooms and changes in the salinity concentration by rain, snow or evaporation. The sea water is heated to 28 °C. and passed

through a biological filter as well as aerated before it is used in any water system. The sea water is supplied from the coastal area of St Andrews, Scotland, UK.

2.1.1.2. Freshwater supply

The source of the freshwater is the main tap water supply system of the Institute of Aquaculture (to see the characteristics of this water for the experimental period, see Appendix II,a). This reliable supply of water is dechlorinated with aeration and kept preheated in a 150 L polypropylene black cylindrical tank (Wizard) with thermostatically controlled electrical heaters before being used in the prawn water systems.

2.1.1.3. Photoperiod and air temperature

The unit is fitted with automatic analogue light timers (Quartz, Sangano) to control the photoperiod, which is adjusted to maintain a 12 : 12 hours (dark : light). The air temperature has an average of 26 °C., with extremes of 21 and 28 °C. and is kept in that range of temperature by all the thermostatically controlled electrical heaters in the different water systems plus two dehumidifiers (HD 602 Hyward). Besides, there is an insulation of fibreglass material in the roof of the unit and blocked up windows that help to prevent heat losses.

2.1.1.4. *Artemia* spp., section

Another facility of the Prawn Unit is the equipment to produce reasonable quantities of *Artemia* spp. It is important to mention here that *Artemia* spp., has been used for several years in the aquaculture industry, as a substitute for natural planktonic diets, not only for *M. rosenbergii,* but for several aquatic species around the world. The aquacultural success of these organisms in feeding the larval stages of aquatic cultured species is because it is possible to keep them for long periods of time as cysts before they are required, as well as because they are relatively easy to culture and provide essential nutrients and high protein levels required by the larvae (Sorgeloos *et al.*, 1977; Tunsutapanich, 1982). Smith *et al.*, (1977) mentioned that the important factor in the consumption of *Artemia* nauplii is the yolk sac still present in the nauplii, rather than the whole animal itself. However, *Artemia* spp. frequently have insufficient levels of long-chain polyunsaturated fatty acids, mainly 20:5 n-3 and 22:6 n-3 (Navarro *et al.*, (1992) which are indispensable for marine animals (Sargent *et al.*, 1997) and since the larvae of *M. rosenbergii* are brackish water organisms, a source of those nutrients should be supplied. In 1982, Watanabe *et al.,* developed a formula to prepare a home made emulsion rich in n-3 polyunsaturated fatty acids, using marine fish oil in its composition (see Appendix II,b), which could be then added to the *Artemia* nauplii rearing system to give them the complete nutritious value required for the larvae of *M. rosenbergii.* The procedure for the enrichment of the *Artemia* spp., is described in section 2.3.2.

The *Artemia* spp. system was basically a series of three polyethylene cylindrical bins with conical bottom (Mailbox 70 L capacity) mounted in an aluminium frame (Plate 1.). These bins were translucent with a white coloration, while the lids were black. Each bin was equipped with a tank connector and a rubber bung at the bottom to allow an easy cleaning routine. The air supply was taken directly from the main system of the Tropical Prawn Unit, through a connection made of a flexible plastic tube divided into three sections. Each section ended in a Polyvinyl Chloride (PVC) pipe extension in order to make it rigid and be able to stand at the bottom of the conical bins. In this way, the aeration was from the bottom to the top of the water column, so that the *Artemia* spp. cysts could be kept in suspension. The air bubbling helped to keep most of the cysts in circulation in the bin. Light was provided directly above the *Artemia* spp. system within a distance of 0.5 m above the surface of the water column. This was because illumination is necessary to obtain optimal results in the hatching rate of cysts (Sorgeloos, 1973). Each bin had a thermostatically controlled aquarium electrical heater set at 28 °C. The salinity of the brackish water prepared every day for the *Artemia* spp. rearing system was in the range of 15 to 18 ‰, since this range of salinity gave a 75 to 80% hatching rate.

Plate 1. Detail of a tank rearing *Artemia* nauplii (left) and daily used equipment for its production (right).

2.1.2. Tropical Aquarium

The Tropical Prawn Unit works in combination with the Tropical Aquarium to maintain the production of freshwater prawns. The Tropical Aquarium is a separate unit within the Institute of Aquaculture at the University of Stirling. This unit is a bigger tropical space (1,500 m^2 of tanks facilities), where several species of different aquatic animals are kept including crustaceans. The freshwater supply, the temperature and photoperiod are the same as those for the Tropical Prawn Unit (sections 2.1.1.2. and 2.1.1.3.). Aeration is supplied by the main air system of the Tropical Aquarium (Single phase D80, Electromotors, air blower). The Tropical Aquarium works as a nursery station for the freshwater prawn postlarvae produced in the Tropical Prawn Unit (all year round). It also sustains a second population of *M. rosenbergii* as a supply of replacement broodstock. This population is firstly grown in communal tanks and then kept individually for future breeding.

2.1.3. Recirculation systems

All the recirculation systems used for the experiments in this work consisted of a 150 L black polypropylene (Wizard) header tank, a holding tank or raceway (home made fibreglass or Fastank ®), a biological filter made up with plastic rings (Mass Transfer International) and a pump. With the exception of the postlarval system, all the systems had a gravel filter (10 mm limestone, Eglinton) to help with the buffer capacity of the system.

2.2. SOURCE OF PRAWNS

The stocks of prawns that are kept in the Prawn Unit are from the same population as those kept at the Tropical Aquarium. They were originally introduced to the Institute of Aquaculture from Thailand in 1985, and since then, the population has been kept in both recirculation systems at the two units. The broodstock kept in the Tropical Prawn Unit were reared in the same conditions as the ones in the Tropical Aquarium, but some organisms in the Tropical Prawn Unit had malformations of some appendages as well as some melanised spots distributed in different parts of their bodies (personal observations). So therefore, the broodstock used in the production of the organisms for the purposes of this work were from the ones kept in the Tropical Aquarium. These prawns were maintained in small populations in two separate Fastank ® raceways (2.8 m L* (length) x 1.5 m W* (wide) x 0.55 m H* (high)) both of them with freshwater recirculation systems (Plate 2.). These recirculation systems had a 2 m high plastic column with a biological filter, instead of a header tank and an external water pump (Beresford PPV41). The two raceways were supplied with air through 8 plastic tubes immersed in the raceways and ending in 12 cm airstones (Algarde) (Plate 3.).

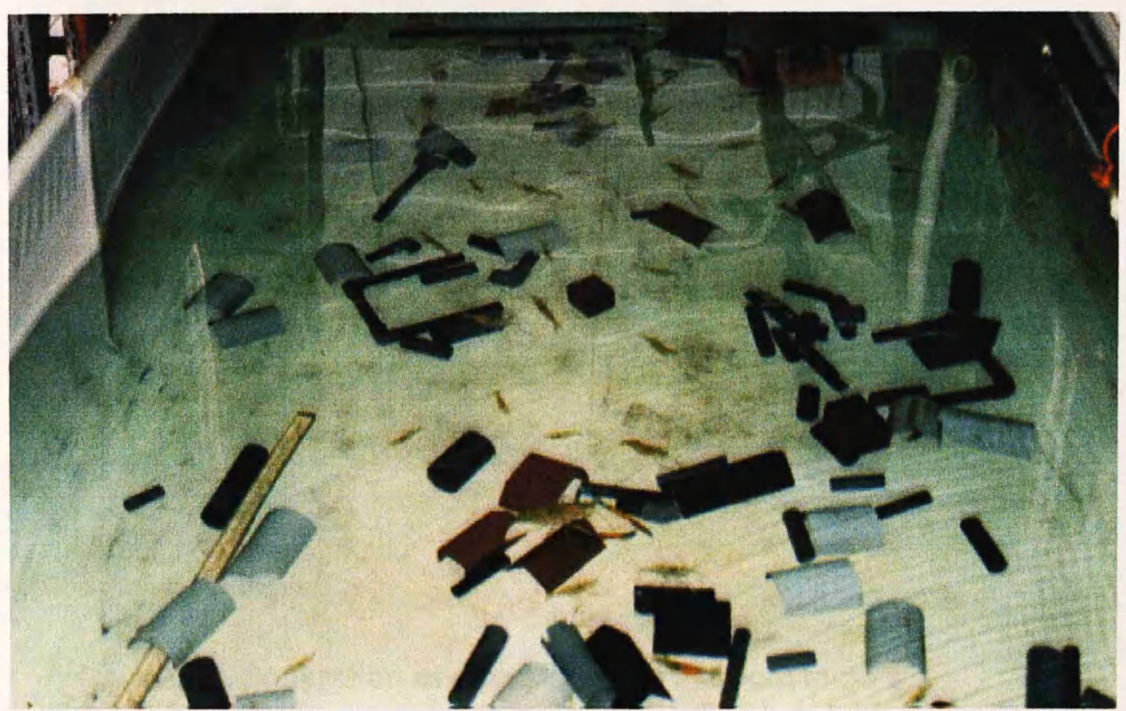


Plate 2. Fastank ® raceway and shelters of different sizes, as part of the freshwater recirculation system for the populations of *M. rosenbergii* held in the Tropical Aquarium.

Plate 3. Recirculation system for ongrowing populations of *M. rosenbergii*.

The two prawn populations were fed with defrosted raw green French beans, alternating with defrosted cooked mussels and raw squid, as well as with artificial trout diets (pellets, fry 0.2 granules, Trouw Aquaculture) on a daily basis (Plate 4.).

Plate 4. Food used for the feeding of *M. rosenbergii*.
From left to right, raw green beans, cooked mussels, raw squid and dry pellets.

2.2.1. Separation of sexually mature broodstock

Mature females could be identified by an orange coloration in the top part of the cephalothorax, between the eyes and the position of the heart, indicating a ripening of the ovaries and they could then be mated at the next moult. Penaeids usually moult during night time (Wassenberg and Hill, 1984) and in this study it was noticed that most of the prawns followed the same behaviour, since most of the old exoskeletons were found early in the next morning after moulting. However, the female prawns were usually collected from the raceway after they were carrying eggs for the first time and this was most likely to be when they were larger than 2 cm carapace length (CL). The male prawns were used for reproduction purposes after they were 3 cm CL and their claws were well developed. Once the prawns were sexually mature, they were individually transferred to a green polypropylene covered rectangular tank (0.55 m L* x 0.36 m W* x 0.24 m H*) in a different recirculation system (Plate 5.), but with the same physico-chemical characteristics as those of the

raceways. Keeping the broodstock individually prevents cannibalism and allows an individual record of moults and growth rate. There were 24 of those rectangular tanks in this system and they were individually aerated via a 6 cm airstone. The water flow rate was 36 L h^{-1} for each tank.

Plate 5. Individual containers for broodstock of *M. rosenbergii*.

2.2.2. Broodstock mating

Once a female prawn completed the pre-spawning moult in its individual tank, a large blue claw mature male was placed into the female tank and left there for about 2 to 4 hours (time to be sufficient for them to mate, personal observations). After that, the male was removed from the female tank and put back into its own tank.

2.2.3. Incubation of eggs

Usually the female started to spawn and laid the eggs on the first four pair of pleopods after 6 hours from the mating time, and sometimes after just 2

or more than 24 hours (personal observations). The incubation period was normally between 17 and 19 days at 28 ± 0.5 °C. The female was transferred into a darkened hatching tank (plastic aquarium, 0.4 m L* x 0.25 m W* x 0.3 m H*) in the Tropical Prawn Unit, after the eggs were of a grey-brownish coloration. This colour was usually present after day 15th, due to the large size of the eyes and consumption of the orange coloured yolk. The water temperature was maintained at 28 ± 1 °C in the hatching tank by an electrical aquarium heater (adjustable Visitherm 300 W) immersed into the tank. The salinity was 12 ‰ and aeration was provided through a 2.5 cm airstone diffuser. Although the photoperiod was 12 : 12, the hatching tanks were darkened with a black plastic, in order to reduce the light and the stress to the female prawn caused by passers-by next to the hatching tanks area. There the female was kept without food until all the larvae were hatched. This period of time was no more than a couple of days if all the eggs were hatched at once, but sometimes eggs were hatching over several days.

2.3. HATCHING OF *Artemia* spp. FOR FEEDING EXPERIMENTAL LARVAE

One of the conical bins, in the *Artemia* spp. system (described in section 2.1.1.5.), was partially filled with 20 L of brackish water (15 to 18 ‰) and then 5 g of dry cysts were added for hatching. The cysts were previously hydrated for a period of 1 h in a conical flask (2 L capacity). During the hydration procedure, the hydrated cysts were sinking to the bottom of the flask

and the dead, as well as the unhydrated cysts were on the surface. The stratum of the surface was then gently discharged by decantation. The remaining hydrated cysts were cleaned with a 10% bleach solution (5% Chloride bleaching agent), for 2 to 3 minutes, in order to kill the possible existent bacteria population on the surface of the cysts. After that, the cysts were rinsed several times with freshwater in a fine sieve (120 µm) and then the possible remaining bleach agent was neutralised with a solution of sodium thiosulphate. The cysts were repeatedly rinsed with freshwater before they were transferred to the conical bin. A 70% hatching rate was attained by the 20th h.

2.3.1. Harvesting the *Artemia* spp.

After the first 24 hours, the light and aeration were switched off and the *Artemia* nauplii were harvested by siphoning the water into a fine nylon sieve (150 µm, mesh size), taking care of not to siphon the surface, but the middle and bottom parts of the water column. The surface of the water column contained the cysts that had not hatched. The hatched nauplii were separated from the unhatched cysts and debris by placing the material collected in the sieve into a conical funnel. Tape and a lid blackened the top and bottom of that funnel, so that only the central section of the funnel was transparent and therefore illuminated when light was applied. The solid particles in the water column would be then sink to the bottom of the funnel and the dead cysts and the ones that did not hatch would float to the surface, meanwhile the swimming nauplii would stay in the middle of the funnel attracted by the light. Another

siphoning of the water was then carried out taking care to collect only the orange newly hatched nauplii from the middle section of the funnel.

2.3.2. Enrichment of the *Artemia* nauplii

The hatched nauplii were enriched by transferring them into a second conical bin filled with 20 L of water of similar conditions as the one prepared for the hatching of the nauplii (see section 2.1.1.5.), and where an enrichment emulsion was then added to the water column, (see Appendix II,b for the formulation of the home made enrichment emulsion). The enrichment to water ratio was 0.001 : 1, so if there were 20 L of water then, 2 mL of enrichment were added. A 500 mL volume of the enrichment was prepared almost every four months in the dry laboratory section of the Tropical Prawn Unit and kept there in a refrigerator at about 3 to 4 °C.

The *Artemia* nauplii were reared in the second conical bin for another 24 hours and then harvested as metanauplii in the same way as the 24 hours nauplii (see section 2.3.1.), in order to eliminate the possible remaining cysts, and then to be fed to the larvae during the experiments. The production of the 24 hours *Artemia* nauplii and the 48 hours *Artemia* metanauplii was done on a daily basis, as well as the cleaning routine. Basically this routine consisted in the cleaning of all the equipment used for the production of the *Artemia* nauplii and metanauplii, including bins, heaters, air-tubing, glassware and sieves to prevent pathogenic organisms from colonising the system. The brand of

Artemia spp. used to feed the larvae in all the experiments was the Supreme 99, San Francisco Bay, from the Great Salt Lake, USA; the batch number was 0027.

2.4. PRODUCTION AND REARING OF LARVAE

The female was removed from the hatching tank after the larvae were hatched, and returned to her previous freshwater tank in the Tropical Aquarium. Every batch of larvae was counted indirectly using an automatic pipette (Pipetman ®, P-Gilson), by taking 1mL sub-samples (ten minimum) of a known volume (usually up to 1 L) of water where the larvae were concentrated. The water was agitated before the sampling with a glass rod in order to get an equal distribution of the larvae in the water column. The mean and standard deviation were calculated to make an estimation of the total number of larvae. Once the total number of larvae was estimated, they were transferred into a recirculation brackish water system. The larvae rearing containers were round polyethylene black bins with conical bottom (70 L capacity), which provided a better distribution of the food and movement of the larvae when aeration and water flow were introduced to the water column (Plate 6.). Furthermore, this

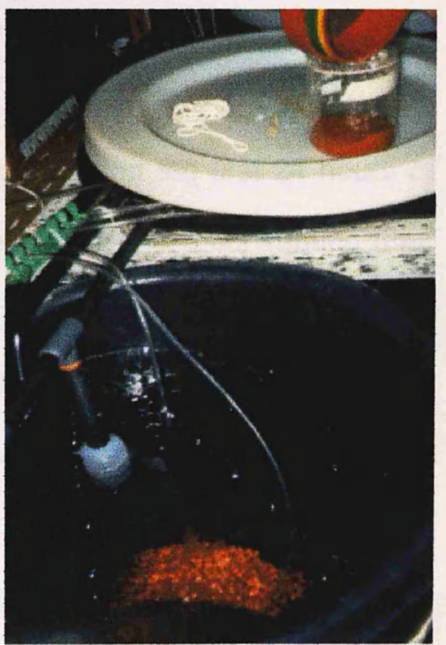


Plate 6. Larval rearing system. Larval container (bottom), *Artemia* nauplii ready to be fed to the larvae (top).

kind of tank reduces the accumulation of waste in contrast with a large bottom tank with multiple dead zones, and allows an easy cleaning routine. New and Singholka (1982) suggest that dark tanks provide better results in the feeding of the larvae. This could be because of the larvae were less stressed by the reduction in light intensity or because the unidirectional light from the top of the tank helped the larvae to see the prey (*Artemia* spp.) passing near by on top of them (Funge-Smith, 1991). The first argument is strengthened by the findings of Moller (1978) that *M. rosenbergii* larval feeding behaviour responds to a chance encounter rather than a visualising and pursuit to capture the prey. However, larvae of *M. rosenbergii* respond positively to light in a similar way as *Artemia* nauplii does (Moller, 1978). Therefore, if the prey and predator are concentrated in the top of the water column, attracted by the light, then there are more chances for the larvae to encounter the *Artemia* nauplii, helping to get a more successful feeding regime. This would then explain the advantage of the dark tanks and the resulting unidirectional light source.

There, the larvae were kept without food during the first 24 hours of their development. During this period of time, the larvae were consuming their own vitellus left from the embryonic development (Ling, 1969). After that, most of the larvae (> 80%) moulted to the second stage of development and then they were daily fed with 48 hours enriched *Artemia* spp. metanauplii only. The salinity, temperature and photoperiod were kept similar to the hatching tank. The conical tanks were totally dried and cleaned every four days, by siphoning the water and retaining the larvae in a 500 μm nylon mesh sieve. All the detritus and the uneaten live or dead *Artemia* spp. were passed through the 500 μm

sieve and collected in a 150 µm sieve. After that, the larvae were placed in the tank and new enriched *Artemia* metanauplii were then added. The larvae were observed every day to evaluate their growth and to identify their developmental stages, using the guide described in 1969 by Uno and Kwon.

2.5. POSTLARVAE REARING

Once the larvae were metamorphosed into postlarvae, they were transferred to a freshwater recirculation system in the Tropical Aquarium.

Each group of newly metamorphosed postlarvae was kept in the same conical tank with the larvae until there was a sufficient number of postlarvae (i.e. between 3000 and 5000) to be transferred to the Tropical Aquarium. This usually took from three to four days. The postlarvae were separated from the larvae in a swirl action system. Such a system was a cylindrical flat bottom bucket with a hole in the centre and an attached overflow pipe. The bucket was installed on top of one of the conical larvae tank. A current of water was applied from one side of the bucket, creating a swirl in the centre, and then falling into the original conical larvae tank (Figure 5.). Once a conical tank containing larvae and postlarvae was emptied, and the larvae and postlarvae were collected as mentioned in section 2.3., the separation system was installed on top of that tank, and the larvae and postlarvae collected were poured into it. The larvae and postlarvae were separated using the positive rheotaxis of the postlarvae, where the postlarvae settled down to the bottom of

the bucket heading up to the current, while the swimming larvae were pulled into the swirl and ending in their original rearing tank (Martinez-Palacios *et al.*, 1985).

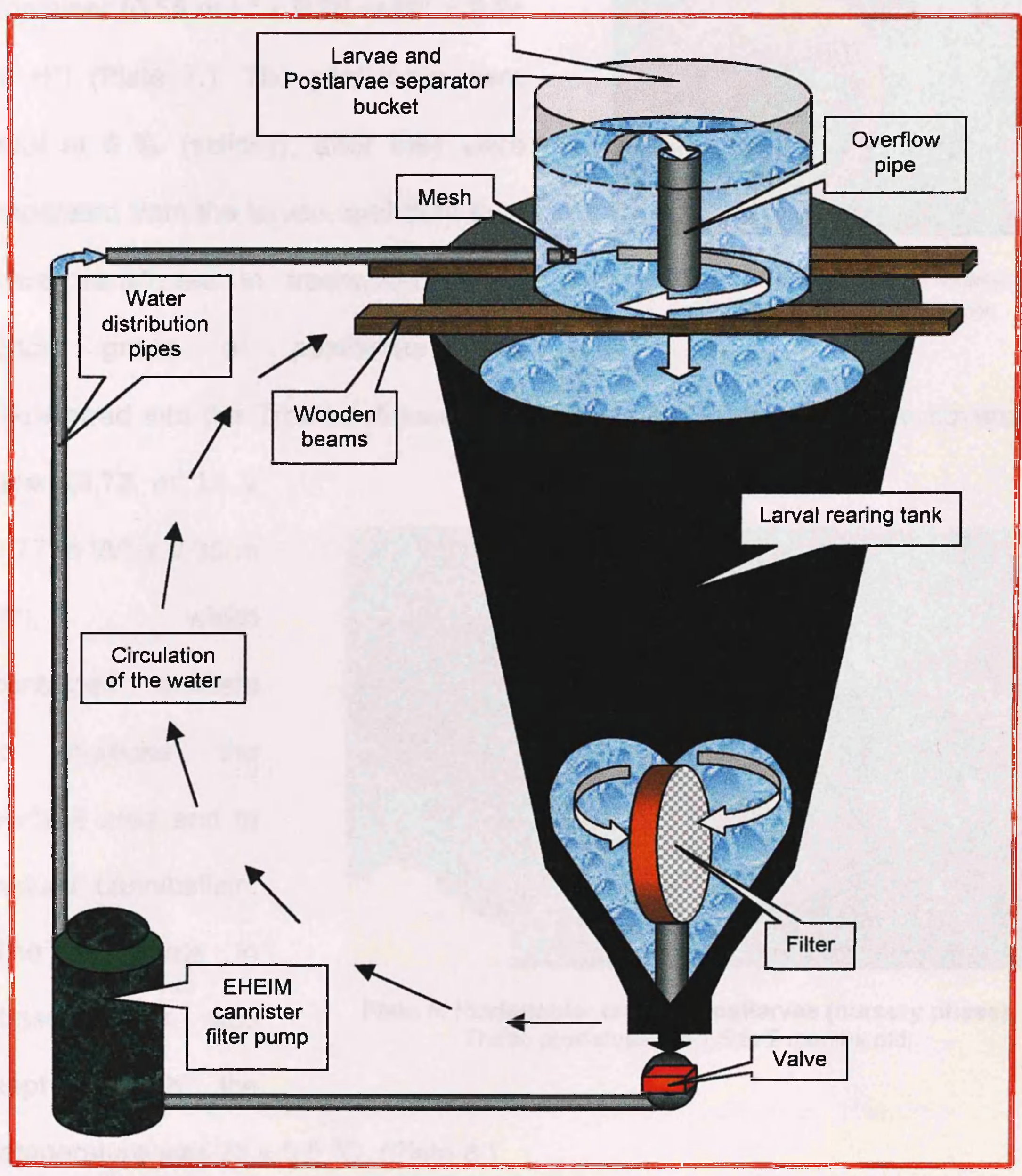


Figure 5. Larvae and postlarvae separator system.

After that, the postlarvae were acclimatised to lower salinity levels from 12 ‰ to 0 ‰. This was done in two stages over 24 hours in a polypropylene container (0.55 m L* x 0.36 m W* x 0.24 m H*) (Plate 7.). The postlarvae were kept at 6 ‰ (salinity), after they were separated from the larvae, and then they were transferred to freshwater tanks. Each group of postlarvae was transferred into the Tropical Aquarium to a rectangular polypropylene covered tank (0.72 m L* x 0.77 m W* x 0.35 m H*), which contained shelters to increase the surface area and to reduce cannibalism. The flow rate in those tanks was kept at 36 L h^{-1}, the temperature was 28 ± 0.5 °C. (Plate 8.).

Plate 7. Acclimation of newly metamorphosed postlarvae.

Plate 8. Rectangular tank for postlarvae (nursery phase). These postlarvae are 1.5 to 2 months old.

The tanks were covered with the lids to prevent them from escaping and to maintain them in dark conditions to induce them to feed more often

(Avendaño Morales, 1994). Aeration was constantly supplied. The postlarvae were fed twice a day with a mixture of flakes of food for tropical fish (Aquarian), and small particles of the same diet as the one described in section 2.2. for the broodstock. The bottom of the tanks was siphoned every day in order to help the biological filter capacity, reduce the production of ammonia, and to avoid a possible blockage of the filter system.

The postlarvae were kept in these tanks until they were needed for the future experiments or until they were big enough to be transferred into the broodstock raceway.

2.6. WATER CHEMISTRY FOR EXPERIMENTAL PURPOSES

2.6.1. Deionized water source

Tap water was passed through a 1 µm polypropylene filter and afterwards it was passed through a deionizer (Permutit, model 3C) to maintain a conductivity of < 0.2 mS cm^{-1}. If the conductivity exceeded that level then, the resin cartridge inside the deionizer was changed for a new one. The deionized water was stored in a polypropylene black tank (Wizard PC50) equipped with an electrical heater (Howden 2 kW, 240 volts) set to maintain a constant temperature of 28 °C. A plastic tube ending in an airstone was introduced to this

tank in order to supply air, and therefore, helping to maintain gas exchange and to homogenise the water column.

2.6.2. Chemicals used in the experimental water

All the chemicals used to create the required total hardness and alkalinity levels were of AnalaR grade (BDH, Poole Dorset) and they were in a powder form. Table 6., shows the chemicals specifications. The chemicals were selected according to the specifications of the HMSO (1969) for the composition of artificial water hardness, but the concentrations of sodium bicarbonate were different (Wurts, 1993), in order to be able to produce water of different total hardness and alkalinity.

Table 6. Chemicals used in the experimental water.

Scientific name	Formula name	Molecular weight
Calcium chloride hexahydrated	$CaCl_2 \cdot 6H_2O$	219.08
Magnesium chloride hexahydrated	$MgCl_2 \cdot 6H_2O$	203.3
Sodium sulphate	Na_2SO_4	142.04
Potassium chloride	KCl	74.55
Sodium bicarbonate	$NaHCO_3$	84.01

Each experiment had different levels of alkalinity and total hardness and therefore different concentration of the different chemicals used in this work. Table 7., shows the different concentration used.

Table 7. Chemicals concentrations.

Alkalinity level		25	50	100	150	200
Chemical in mg L^{-1}	$NaHCO_3$	36	73	147	231	316
Total hardness level		**25**	**50**	**100**	**500**	**1000**
Chemicals in mg L^{-1}	$CaCl_2 \cdot 6H_2O$	27.375	54.75	109.5	547.5	1095
	$MgCl_2 \cdot 6H_2O$	25.375	50.75	101.5	507.5	1015
	Na_2SO_4	178.5	178.5	178.5	178.5	178.5
	KCl	7.1	7.1	7.1	7.1	7.1

2.7. WATER QUALITY PARAMETERS

2.7.1. Temperature

The temperature was kept at 28 ± 1 °C in all the experiments by introducing glass aquarium electrical heaters (Visitherm 300 w) into the raceways and hatching tanks, and electrical heaters (Howden 3 kW) in the header tank of the larvae rearing system and broodstock systems. The temperature was measured in a daily basis with glass general purpose thermometers (BDH) with a scale in a range of -10 to +50 °C.

2.7.2. Ammonia

Total ammonia was monitored on a daily basis with Tetra Aquarium total ammonia kits (NH_3-N-test, Animal House, Ltd.) for simplicity. An

autoanalyser (Technicom ®, Sampler IV, Gradko International Ltd.) was used to measure the levels of total ammonia (for greater accuracy) during at least one whole week of the experimental time, and then once per week in each experiment. The method used for this automatic determination uses salicylate chemistry for ammonia, described in the Standard Committee of Analysis. HMSO, 1982.

2.7.3. pH

The pH was measured every day with a pen-pH meter (Waterproof Hanna Instruments, pHep ® 3) and once per week with a CD720 WPA potentiometer, for maximum precision.

2.7.4. Dissolved oxygen

An oxygen meter (DO_2 Meter, Jenway 9071) was used to measure the dissolved oxygen in the water column. This was monitored every day during the first week of each experiment and due to the low variability then, was done every four days in each treatment of the three experiments. Usually the dissolved oxygen levels were constantly higher than 70% in all the experiments.

2.7.5. Salinity

A hand refractometer (CSP 0-100 ‰) was used to measure the salinity every day in the brackish water systems (12 ‰). Losses by evaporation (5 to 10 L day^{-1}) were substituted daily with warm freshwater from a reservoir tank, in order to maintain the salinity required.

2.7.6. Alkalinity

Initially the alkalinity was monitored every other day (in order to estimate the increases by evaporation), but after the first experiment it was seen that the increment of this parameter in the water was not significant. Therefore, it was decided to measure it every week and whenever there was a water change. The procedure to measure the alkalinity by the titration method is described in Stirling (1985) and Clesceri *et al.*, (1989) (Appendix II,c).

2.7.7. Total hardness

Total hardness was measured at the same frequency and on the same days as the alkalinity. The same authors that describe the alkalinity titration method describe the method for total hardness titration (Appendix II,c).

2.8. STATISTICAL ANALYSIS OF THE RESULTS

The homogeneity of variance and the normality of the data were tested before any further analysis of the experimental data (in all the experiments) was carried out. Transformation of the data was done to analyse the survival percentages, however, when the survival percentages were analysed, the results of the analysis were similar when transformed and without transformation. A one way analysis of variance (ANOVA) was used for the experiments which only had one variable (alkalinity), but when there were two of them (alkalinity and total hardness) a two way ANOVA was applied. A Tukey test (multiple comparison test) was applied for a one way ANOVA in order to identify the differences among the treatments. When there were differences in a two way ANOVA a Bonferroni method was used to help to identify the differences in the multiple comparisons test (Howell, 1997). "T" test was used to compare the mean of two different sets of data. For the analysis of the second part of the study, general linear methods were applied and multiple comparisons were applied to identify the differences among the individuals, replicates and treatments, using the Bonferroni method (Howell, 1997). The results were computed with the help of the Minitab 11.12 statistical program, and the statistical procedures described in the books of Bliss, 1970; Sokal and Rohlf, 1987; Winer *et al.*, 1991; Mead *et al.,* 1993; Watt, 1993; Fowler and Cohen, 1996; Howell, 1997 and Zar, 1999.

CHAPTER 3

3. RESPONSES OF *M. rosenbergii* POSTLARVAE TO DIFFERENT LEVELS OF ALKALINITY AND TOTAL HARDNESS

3.1. INTRODUCTION

Ionic transport within crustaceans has been studied in detail. It is useful to review some of these studies in relation to the metabolic functions of *M. rosenbergii*.

3.1.1. Brackish water in the culture of *M. rosenbergii*

The early studies on the osmoregulatory capacity of *M. rosenbergii* were orientated to find out the salinity requirements of the species during the different stages of its development. In 1957, John indicated the presence of *M. rosenbergii* in both brackish and freshwater in India, commenting on the changes in salinity tolerance of the species and the necessity of larvae for a 1 to 1.5% (0.35 to 0.5 ‰ respectively) of sea water at hatching. Similarly, Ling and Merican (1961) reported of the regular presence of *M. rosenbergii* in brackish waters of Malaysia. Ling in 1962 and 1969, studied the development of larvae of *M. rosenbergii*, finding that brackish water between 20 and 40% of sea water (7 and 14 ‰ respectively) was indispensable for their development specially during the change from stage I to stage II of the larval development, whilst Uno and Kwon (1969) utilised a salinity of 30% of sea water (10.5 ‰) in their

experiments to promote the development of the larvae. George (1969) indicated that larvae of *M. rosenbergii* could tolerate salinities between 5 and 20 ‰.

Studies by Singh (1980) showed that although postlarvae of *M. rosenbergii* were able to grow in water with salinity similar to its isosmotic point, their best growth rate was achieved in freshwater. Sandifer *et al.*, (1975) also studied the performance of postlarvae *of M. rosenbergii* in brackish water finding that mortality started to occur at 25 ‰, and increased rapidly at levels of 30 ‰ or above, when the isosmotic point of the postlarvae was 17 ‰. They mentioned that an acclimation to salinity, by gradually increasing the salinity instead of an abrupt change, could extend the time of the lethal effect of salinity at levels up to 35 ‰. More specific studies on the physiology of the organisms were conducted in relation to salinity as well, for instance, the work of Mykles and Ahearn (1978) demonstrated that the midgut of *M. rosenbergii* has an important role in the water transport during the pre-moult stage (to increase the size of the prawn by adding water to the tissues). Later on, Ahearn and Kullama (1984) indicated that the intestinal water transport as well as the transport of sodium (Na^+) and chlorine (Cl^-) which act as absorptive ions and potassium (K^+) as secretory, were affected by allosteric proteins (theopylline and cAMP). The activation of those proteins depended on other agents such as exogenous Ca^{2+}, this ion was incorporated in the protein as a third binding ligand, helping the protein to reduce or eliminate coupled Na^+ and Cl^- influx into the cell from the gut lumen and at the same time controlling the water flow. Castille and Lawrence (1981) studied the effect of salinity on the osmotic Na^+ and Cl^- concentration in the haemolymph of *M. rosenbergii*, finding that this species is

an effective hyperosmotic and hyperionic regulator of its blood osmotic pressure in very dilute media (< 14 mOsm kg^{-1}). Stern *et al.,* (1987) investigated the osmotic and ionic regulation of *M. rosenbergii* adapted to varying salinities (0 to 24 ‰) and ion concentrations, concluding that the species is capable of maintaining and regulating its haemolymph osmolality and ionic composition in freshwater and in water with concentrations approaching iso-osmoticy.

Similar studies have been undertaken but with a different aim, that of determining where prawns would grow. There are large amounts of brackish water existent in different tropical and subtropical countries, which could have potential for the development of the prawn industry. Popper and Davidson (1982) experimented with the growth of *M. rosenbergii* postlarvae in salinities between 0 and 25 ‰ in Western Samoa, finding that salinities of 10 to 15 ‰ did not adverselly affect the growth of the organisms. Similar results were achieved in brackish waters of South Carolina, USA by Smith *et al.,* in 1982, where they mentioned that postlarvae and juveniles of *M. rosenbergii* were growing similarly in both fresh and brackish water at up to 10 ‰. Howlader and Turjoman (1984) did not attribute the decreasing growth rate of their experimental prawns to the salinity (1.6 ‰) of Al-Hassa oasis water in Saudi Arabia, but to the high levels of hardness (600 to 1000 mg L^{-1} as $CaCO_3$). Stern *et al.,* (1984) studied the effects of salinity on the oxygen consumption when juvenile prawns were reared in different Israeli saline waters. They mentioned that no variation was observed between the oxygen consumption in high and low salinity waters, except for the water of Yahel (2 ‰), where the calcium was high compared with the other dilute water media and as a consequence, that

induced a less active Na^+ uptake and suggesting a less metabolic oxygen consumption. El-Gamal (1985) and Sadek and El-Gayar (1995) studied the possibility of culturing *M. rosenbergii* in brackish waters of Egypt, finding that this industry could be profitable due to the growth rate of the prawns in the Egyptian brackish waters (4.5 to 4.6 mg day^{-1}, for postlarvae). Sadek and El-Gayar (1995) mentioned that two crops of marketable prawns can be produced in brackish water of Egypt in on-growing ponds with salinity levels ranging between 4 and 6 ‰, hardness between 900 and 1500 mg L^{-1} as $CaCO_3$ and alkalinity between 115 and 130 mg L^{-1} as $CaCO_3$.

3.1.2. Ionic regulation in *M. rosenbergii*

However, as mentioned earlier, apart from Na^+ and Cl^- (salinity) other ions could affect the performance of *M. rosenbergii,* which have been studied in relation to their metabolic functions. From the early larval stage, different ions have been investigated to find out their requirements or disadvantages in the development of the species. Funge-Smith (1991) investigated the requirements of different ions in simplified sea water culture media for the development of *M. rosenbergii*. He found that a simplified sea water could be used in the production of the larvae, provided bromide and strontium were added to the composition. In 1989, Liao and Hsieh, found that copper, cadmium and zinc were more toxic to larvae of *M. rosenbergii* as the developmental stages of these organisms were advancing, affecting their growth and survival.

Some other studies have been conducted to estimate the viability of water sources containing or lacking certain elements, for instance, Baker (1989) found that loss of live weight and reduction in the exoskeleton size increments of *M. rosenbergii* were caused by concentrations of aluminium ions at 69 μg L^{-1} when the pH was neutral. The studies made to evaluate waters with different salinity and some other ions are of great interest for the potential of the species to be developed in brackish waters, where climate conditions are adequate for its culture. However, large freshwater resources are available in several countries around the tropical belts, which are of interest to develop the prawn industry. One of the main constraints of these water sources is the differences in calcium and carbonates in the water, which are two important elements for the development of the species.

3.1.2.1. Effects of calcium carbonate on *M. rosenbergii* development

Some authors have dealt with the effects of hardness in the survival, growth rate and shell mineralization of the freshwater prawn *M. rosenbergii*. However, as it has been described earlier (section 1.5.2.3.1.), there are differences in the concentration of the hardness and alkalinity (particularly calcium and carbonates) depending on the geochemical origin of the water supply, which are not taken into consideration in some of these works, regarding ionic effects in the prawns. Brown *et al.*, (1991) found a better growth rate of *M. rosenbergii* in low levels of hardness (< 53 mg L^{-1} as $CaCO_3$) and

Cripps and Nakamura (1979) reported that the growth rate of *M. rosenbergii* was negatively affected by hardness levels increasing from 65 to 500 mg L^{-1} as $CaCO_3$. However, these works do not distinguish between the hardness and alkalinity in their experimental methodologies. Although those results are in accordance with Vasquez *et al.,* (1989) which found a similar inhibition of the growth rate of *M. rosenbergii* in water with high levels of hardness (225 to 450 mg L^{-1} as $CaCO_3$), they do not agree in the survival reported. Cripps and Nakamura (1979) did not report a negative effect of high hardness in the survival of their experimental prawns (adults), nor did Vasquez *et al.,* (1989) with postlarvae. In contrast, Brown *et al.,* (1991) reported a total mortality of the experimental prawns when the level of hardness was greater than 300 mg L^{-1} as $CaCO_3$. The results of Sukadi (1989), support this: in that he reported a decreasing survival of the prawns as the environmental hardness increased from 18 to 300 mg L^{-1} as $CaCO_3$.

Cripps and Nakamura (1979) used adult organisms in their experiment, and previously they found that high mortality occurred in larvae of the species when cultured in ponds with high calcium carbonate content (305 to 638 mg L^{-1}). It has been found that adults and even juveniles of *M. rosenbergii* are more tolerant to changes in hardness than postlarvae (Sukadi, 1989; Latif, 1992). Therefore the better survival in their study is presumed to be due to the size and age of the organisms, although Howlader and Turjoman (1984), found that high hardness (600 to 1000 mg L^{-1} as $CaCO_3$) increasingly affected the growth as the prawns grew. In the study of Vasquez *et al.,* (1989) the alkalinity was always low (10 mg L^{-1} as $CaCO_3$) and this was presumably keeping a safe

level for the survival of *M. rosenbergii* postlarvae, whilst in the study of Brown *et al.,* (1991) the alkalinity level was the same as the total hardness and, therefore, increased as the hardness increased. The findings of Bartlett and Enkerlin (1983), reported that high levels of hardness between 940 and 1060 mg L^{-1} as $CaCO_3$, did not have a negative effect either in the growth or the survival of *M. rosenbergii*, provided that the alkalinity levels were low (58 and 86 mg L^{-1} as $CaCO_3$). Similarly, Latif (1992), reported that high levels of alkalinity (250 mg L^{-1} as $CaCO_3$) in combination with high levels of total hardness (1000 mg L^{-1} as $CaCO_3$) negatively affected the growth rate in both postlarvae and juveniles of *M. rosenbergii*. Although it has been reported that hardness is not as important as alkalinity in fish culture, and that the higher the alkalinity (8 to 120 mg L^{-1} as $CaCO_3$) the better the productivity in fish ponds (Moyle, 1945 and 1946; Maris, 1966, both cited in: Boyd, 1982), it seems that apparently that is not the case for the culture of *M. rosenbergii*, since the calcium carbonate has a direct effect in the performance of the prawns regardless of the productivity of the water in the ponds.

It is clear that a controversy exists as to what constitutes an optimal and safe level of total hardness in combination with alkalinity. Therefore, it is important for the general knowledge of the species and an optimisation in the use of suitable sources of water for its culture, to reinforce the data and to give a better range of total hardness and alkalinity required for its metabolism. These experiments will investigate the survival, growth rate and exoskeleton mineralization of postlarvae of *M. rosenbergii*, under wide ranges of total

hardness (25 to 1000 mg L^{-1} as $CaCO_3$) and alkalinity (25 to > 250 mg L^{-1} as $CaCO_3$).

3.2. METHODOLOGY

3.2.1. Experimental design

Investigations with postlarvae of the freshwater prawn *M. rosenbergii* were conducted at different levels of alkalinity and total hardness. With the limitations of space and time required to run the experiments it was necessary to carry out the investigations in different sets of experiments. The first experiment investigated alkalinity and total hardness from low to moderate levels (25, 50 and 100 mg L^{-1} as $CaCO_3$). The second investigated from moderate to high levels (100, 150 and 200 mg L^{-1} as $CaCO_3$ for alkalinity and 100, 500 and 1000 mg L^{-1} as $CaCO_3$ for total hardness). The moderate levels (100 mg L^{-1} as $CaCO_3$) were repeated, in both experimental parts, as a way of allowing comparisons to be made between them.

3.2.2. Experimental system facilities

The experimental facilities employed in these experiments were the result of modifications to preliminary experimental trials, described in Appendix

I,a, as many physicochemical constraints were encountered before the actual experiments took place.

3.2.2.1. Flow through water system

There were nine flow-through water systems and all of them were built similarly to each other (Figure 6.).

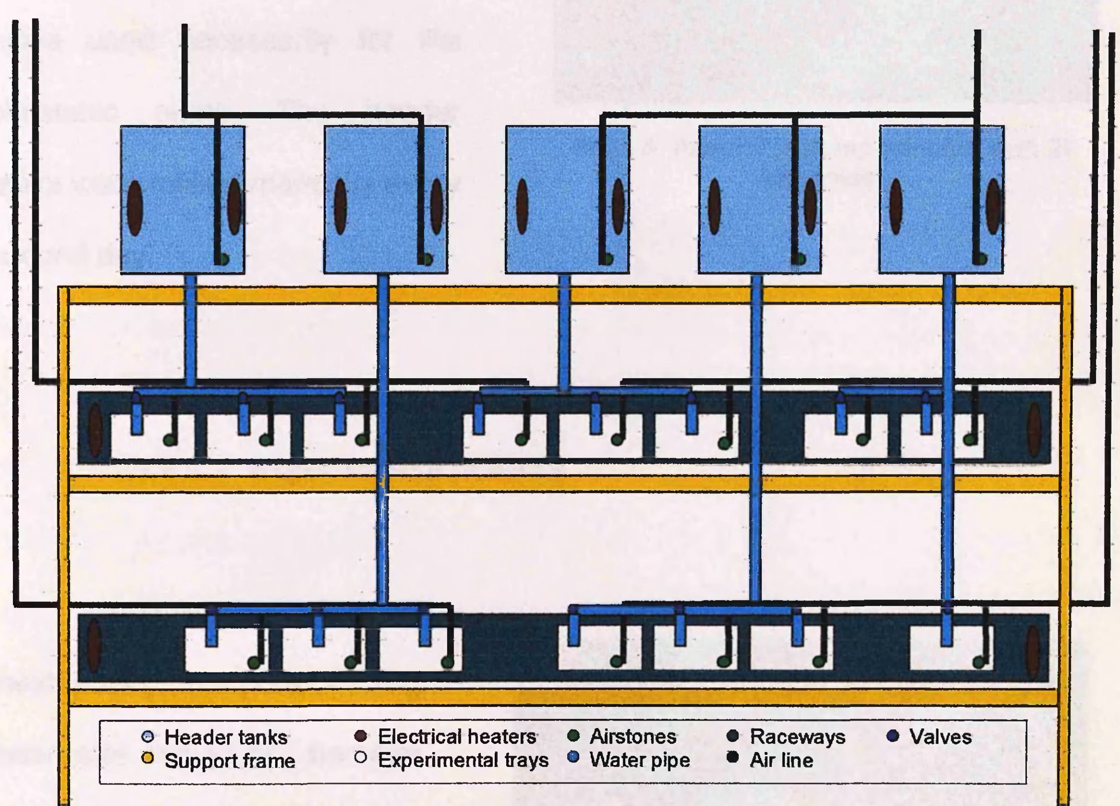


Figure 6. Representation of five flow through water systems.

Each system had a header tank and three white polypropylene containers (Mailbox, 15 L capacity). The water flow of the nine systems was

controlled at the same time by a peristaltic pump (Watson-Marlow Ltd) renewing 100% of the water within 24 hours. (Plate 9.). There were three plastic tubes (3 mm diameter) that drove the water, by gravity, from each of the header tanks. Those plastic tubes were connected to PVC "manifold" tubes used necessarily for the peristaltic pump. The header tanks were refilled manually every second day.

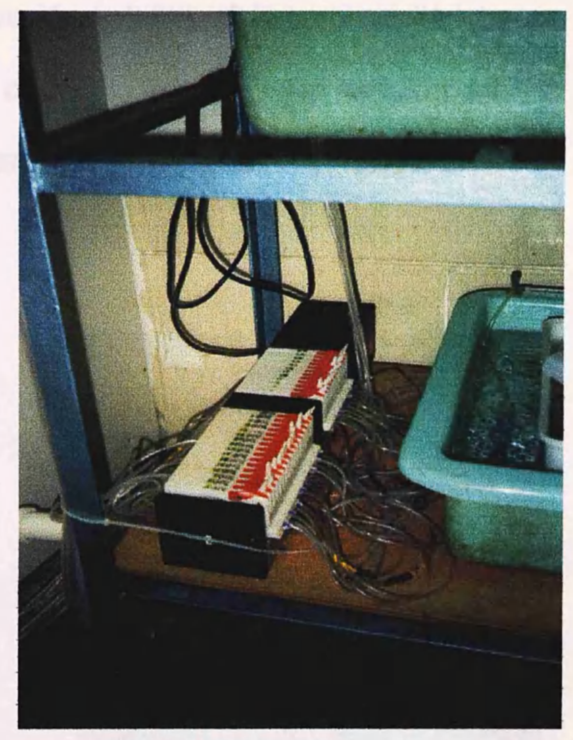


Plate 9. Peristaltic pump adapted with 27 channels.

3.2.2.2. Experimental holders

Three cylindrical mesh pots (3 mm pore opening mesh size and 11 cm diameter x 17 cm H) were allocated to each container (Plate 10.) in order to have three replicates per container and three containers per treatment. The

Plate 10. Container with three meshpots for individual postlarvae.

containers were partially filled to a specific volume of 8 L, controlled by an overflow device. Each container had an airstone to supply aeration and to maintain a homogeneous water medium in the tanks, as well as to sustain a constant gas exchange into the system, for example the removal of carbon dioxide and supply of dissolved oxygen. All the containers were set into fibreglass raceways (Plate 11.), which were partially-filled with water at 28 °C. This was done in order to maintain a constant temperature. Three different raceways were used in different iron frames, due to the limitations in space at the Tropical Prawn Unit. Two thermostatically-controlled electrical heaters situated in each raceway heated the water.

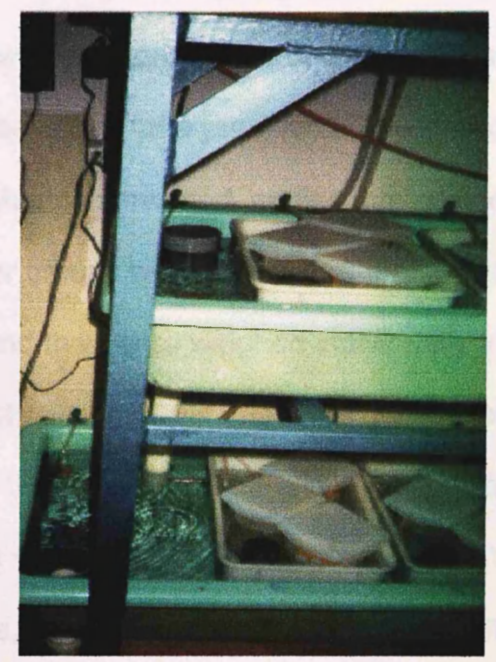


Plate 11. Detail of two raceways used to keep the temperature homogeneous.

3.2.3. Selection of experimental postlarvae

As explained in section 1.5.2.1.1., it is clear that there is strong growth variability among individuals of *M. rosenbergii* during their whole life cycle. Therefore, postlarvae used in this experiment were collected from the

middle cohort, within each experimental population, to avoid or at least to diminish the effect that growth variability could have over the results of these studies. At 45 days post-metamorphosis the three different weights for the three cohorts were similar in the different populations, being < 0.1, 0.1 to 0.2 and > 0.2 g for the small, medium and large prawns respectively, regardless of their parental history (personal observations). It was necessary to have animals of at least 0.1 g so that the carapaces could be easily analysed for calcium and magnesium. Therefore, from the three cohorts mentioned earlier the middle group was selected, and the other two major groups were rejected. A total of 81 postlarvae were used in each experiment and they were weighed individually to the nearest 0.001 g in a digital balance (Mettler AJ100). The initial postlarval weight in grams for both experiment I and II was 0.14 ± 0.03 and 0.17 ± 0.03 respectively. Each organism was captured with a 10 x 10 cm nylon mesh (1 mm, pore size) and immediately afterwards, it was gently blotted dry with soft absorbent tissue (for approximately 5 seconds). Then it was weighed in a pre-weighed plastic tray (Fisher Instruments) and allocated to an individual cylindrical mesh pot. The mesh pots were covered with plastic trays in order to prevent the organisms from escaping.

After selection, all the 81 postlarvae in their individual mesh pots were transferred into the nine open flow water systems (9 postlarvae per system).

3.2.4. Physicochemical parameters of the culture media

All the water parameters like pH, ammonia, dissolved oxygen, temperature, alkalinity, and total hardness, were monitored as described in section 2.7.

The two experiments were kept under similar conditions of dissolved oxygen, temperature and ammonia (Table 8.).

Table 8. DO2, temperature and total ammonia variations for both experiments.

Experiment	Dissolved oxygen (% of saturation)	Temperature (^oC)	Total ammonia (mg L^{-1})
I	84 – 94	27.5 - 28.5	< 0.3
II	85 – 96	27.6 - 28.4	< 0.3

The mean value of total ammonia measured in the autoanalizer was 0.1 ± 0.01 mg L^{-1} and a maximum nitrite level of 0.008 µg L^{-1}.

Those variables were among the optimum required for the species (Wickins 1976a; Nghia, 1991; Straus *et al.,* 1991). The photoperiod was 12:12 hours dark : light and the flow rate was 180 mL h^{-1} giving with this a total water change in the containers every two days.

3.2.5. Alkalinity and total hardness concentrations

The postlarvae were allocated to different water culture media, the composition of which was prepared according to the levels of total hardness and alkalinity required for each experiment.

The concentrations of total hardness and alkalinity were as shown in Table 9. for both experiments I and II. Those concentrations were arranged to create a factorial design of 3 x 3, giving a total of 9 treatments, in each of the two experiments.

Table 9. Total hardness and alkalinity concentrations required for each experiment.

Experiment	Alkalinity in mg L^{-1} as $CaCO_3$	Total hardness in mg L^{-1} as $CaCO_3$
	25	25
I	50	50
	100	100
	100	100
II	150	500
	200	1000

3.2.6. Acclimation of experimental postlarvae

The first week of each experimental time was used as an acclimatisation period for both experiments, starting after the larvae were transferred to their respective treatments. The mortality of the animals was not

counted during this first acclimatisation week, and if mortality occurred then all the dead postlarvae were substituted by new ones. All the postlarvae were weighed again after the acclimatisation period, before the start of the experimental time.

3.2.7. Experimental management

Feeding of the postlarvae was done in a similar way as described in section 2.2., but instead of commercial trout pellets, a home made artificial food was given (To see the proximal analysis of the diet see Appendix III,a) which had a smaller particle size. However, the cleaning procedure was different and that was done, by changing the dirty containers in all the different treatments for clean containers every week. The water flow used was enough to change the water of the containers every two days, but still that water flow was not strong enough to remove small waste particles that accumulated in the bottom of the containers and around the overflow pipe. Whenever a set of containers (three for each treatment) was ready to be changed, a clean set of containers was filled with water from the header tank of its respective treatment. Afterwards, the three mesh pots of a container were transferred to the clean and previously filled one. The transfer of the mesh pot containing the postlarvae was done in the minimum time possible (3 to 5 seconds) in order to avoid too much stress for the postlarvae. Besides this weekly routine, there was a daily cleaning routine, prior to feeding, where all the mesh pots were siphoned to remove all the faeces and the old uneaten food.

3.2.8. Moult collection

The mesh pots were checked twice every day during the cleaning and feeding routines, to collect every single cast carapace and record the moulting date as well as the general health state of the postlarvae.

3.2.8.1. Carapace measurement and storage

All the cast carapaces that were collected were immediately measured with a dial calliper (RS Baty Component) at the nearest 0.1 mm. The measurements were from the middle of the posterior orbital margin to the posterior mid-dorsal margin in each carapace. Once they were measured, they were rinsed with distilled water, and then dried at room temperature (25 °C) for one day. Afterwards, they were storage in plastic vials (Life Sciences Micro-centrifuge tube1.5 mL) for subsequent calcium and magnesium analyses.

3.2.9. Removal of the intermoult carapaces

Both experiments were run until all the surviving postlarvae were in their sixth moult cycle after the experiments were started. The first moult was not counted for the experimental purposes, because, any cause of stress at the beginning of the experiment (like the selection operation from the Tropical Aquarium (section 3.2.3.)) could affect their moult cycle. Each postlarva was

killed in the intermoult period, after they completed their fifth experimental moult, (the intermoult period was taken to be half of the time of the previous moult cycle). This was done by blotting them dry with a soft tissue, weighing them to the nearest 0.001 g and afterwards freezing them for five to ten minutes in order to ensure that they were completely anaesthetised. After that, the intermoult carapace was dissected out, taking care of not to leave any soft tissue attached to it. The intermoult carapaces so obtained were then measured in the same way as the cast carapaces in section 3.2.8.1. After taking their measurements, they were rinsed with distilled water and left to dry at room temperature (20 °C) for 24 hours in plastic vials, for subsequent calcium and magnesium analyses.

3.2.10. Calcium and magnesium analyses of the cast and intermoult carapaces

Both cast and intermoult carapaces were dried at room temperature (20 °C). The carapaces were dried again after the last intermoult carapace was dissected, they were placed in borosilicate glass test tubes (Corning) held in an aluminium rack (Denley) and then left overnight in an oven (Gallenkamp GAOVE300) at 100 °C.

Once the carapaces were dried, they were weighed in a digital balance (Mettler AJ100). Immediately afterwards, they were placed on a heater (Techne dry-block, 08-3H) in screw cap test tubes, where a 5 mL volume of

concentrated nitric acid (69% BDH aristar grade) was added to digest the carapaces. The tubes were heated overnight at 100 °C under a fume cupboard protection.

The resulting solution kept in plastic bottles was diluted at 25 mL by adding distilled water. This dilution was mixed in a ratio of 1 mL of lanthanum chloride at 5% per 4 mL of sample. Lanthanum chloride ("spectrosol" grade) was used to prevent ionic interference when reading the samples in an atomic absorption spectrophotometer (Perkin Elmer, model 2280). The spectrophotometer was calibrated with 2 and 4 mg L^{-1} calcium and 0.1 and 0.3 mg L^{-1} magnesium standards. A 423 and a 285 wave length lamps were used in the spectrophotometer to measure the calcium and magnesium concentrations respectively.

3.2.10.1. Description of the measurements used for the carapace mineralization

- *Length-specific dry weight of carapace* = $\dfrac{\text{Dry Weight of carapace (mg)}}{\text{Length of carapace (mm)}}$

- *Calcium content in the carapace* = $\dfrac{\text{Weight of calcium in the carapace (mg)}}{\text{Dry weight of the carapace (g)}}$

- *Length-specific calcium content* = $\dfrac{\text{Weight of calcium in the carapace (mg)}}{\text{Length of carapace (mm)}}$

- *Magnesium content in the carapace* = $\dfrac{\text{Weight of magnesium in the carapace (mg)}}{\text{Dry weight of the carapace (g)}}$

- *Length-specific magnesium content =* $\dfrac{\text{Weight of magnesium in the carapace (mg)}}{\text{Length of carapace (mm)}}$

3.2.11. Third experimental trial (extreme alkalinity levels)

After the two experiments previously described, a third experimental trial was conducted in order to clarify the trend of the results obtained after the exposure of the postlarvae to high concentrations of total hardness and alkalinity in the culture media of the second experiment. The average initial weight of the experimental postlarvae used during this trial was 0.18 ± 0.02 g.

3.2.11.1. Physicochemical parameters and management

There was the same 12 : 12 hours, dark : light photoperiod as in the previous experimental trials. Dissolved oxygen fluctuated between 86.5 and 94% of saturation, the temperature between 27.4 and 28.4 °C. Whilst the total ammonia was always maintained below 0.3 mg L^{-1}. The food, feeding and cleaning procedures were administered exactly in the same way as in the previous two experiments, as well as the collection of carapaces and mineralization analyses. The only difference between this experimental trial and experiments I and II, was the increase of the alkalinity concentrations to up to 500 mg L^{-1} as $CaCO_3$, and, similarly to the first and second experiments, a

treatment containing alkalinity and total hardness of 100 mg L^{-1} as $CaCO_3$ was conducted as reference.

3.3. RESULTS

3.3.1. Alkalinity and total hardness

The concentrations of alkalinity and total hardness were different for each experimental trial (Table 10.). The pH varied in consequence of the changes in the concentration of $NaHCO_3$ added to the water in spite of the two buffers used (KCl and Na_2SO_4) but only within a range of 8 to 9.

Table 10. Mean values of experimental pH, alkalinity and total hardness, plus and minus a standard deviation (n = 8).

Experiment	pH	Alkalinity (mg L^{-1} as $CaCO_3$)		Total hardness (mg L^{-1} as $CaCO_3$)	
		Required	Obtained	Required	Obtained
I	8.1 ± 0.2	25	28.1 ± 1.9	25	27.3 ± 2.5
	8.1 ± 0.2	25	26.1 ± 0.4	50	54 ± 3.5
	8 ± 0.2	25	25.9 ± 0.5	100	98.7 ± 9.1
	8.4 ± 0.2	50	54.5 ± 1.8	25	27 ± 1.7
	8.4 ± 0.2	50	49.2 ± 0.8	50	54.7 ± 1.5
	8.3 ± 0.2	50	48.8 ± 0.9	100	99 ± 4.6
	8.7 ± 0.2	100	102.3 ± 6.1	25	27 ± 1
	8.6 ± 0.2	100	97.6 ± 0.9	50	49.7 ± 2.1
	8.6 ± 0.2	100	98.3 ± 1.6	100	96.7 ± 2.3
II	8.5 ± 0.1	100	102.8 ± 3.3	100	109.2 ± 5.9
	8.5 ± 0.1	100	103.2 ± 2.5	500	538.4 ± 26.7
	8.4 ± 0.1	100	105 ± 3.8	1000	1023.6 ± 45.6
	8.6 ± 0.2	150	153.7 ± 6.9	100	110.4 ± 7.1
	8.6 ± 0.1	150	150.7 ± 7.6	500	528 ± 23.2
	8.5 ± 0.1	150	137.7 ± 11.3	1000	1027 ± 53.4
	8.8 ± 0.2	200	212.3 ± 10.1	100	104.8 ± 3.3
	8.7 ± 0.1	200	192.5 ± 9.8	500	515.2 ± 12.5
	8.8 ± 0.1	200	187 ± 15.5	1000	1006.8 ± 42.7
III	8.5 ± 0.1	100	107 ± 3.2	100	106.3 ± 1.5
	8.4 ± 0.2	100	85.3 ± 2.8	1000	1069.5 ± 72
	8.9 ± 0.2	250	252.1 ± 18.5	100	95.8 ± 2.3
	9.1 ± 0.3	500	512.9 ± 21.2	100	89 ± 12.8
	9 ± 0.1	500	371.3 ± 47.8	500	362.8 ± 56.5
	8.9 ± 0.2	500	266.6 ± 33.4	1000	851 ± 57

3.3.2. Growth rate

The growth rate of the postlarvae of the three experiments is shown in Table 11. This shows that the growth rate of the postlarvae from the first experiment (lower concentrations of alkalinity and total hardness) was higher in all the different treatments as compared with those of the second experiment (moderate to higher concentrations of alkalinity and total hardness), with the only exception of the control treatment (100 mg L^{-1} as $CaCO_3$ for both alkalinity and total hardness).

Table 11. Growth rate (mg day^{-1}) of postlarvae of *M. rosenbergii*.

Experiment	^ Alkalinity (mg L^{-1} as $CaCO_3$)	^ Total hardness (mg L^{-1} as $CaCO_3$)					
		25	50	100	350	500	1000
I	25	6.1 ± 1.9^{a} (n = 5)	6.9 ± 1.2^{a} (n = 8)	6.2 ± 0.8^{a} (n = 7)			
I	50	5.8 ± 1.2^{a} (n = 9)	5.8 ± 2.2^{a} (n = 8)	5.7 ± 1^{a} (n = 6)			
I	100	5.6 ± 1.7^{a} (n = 7)	5.2 ± 1.1^{a} (n = 8)	5.4 ± 1.3^{a} (n = 6)			
II	100			6 ± 1.6^{b} (n = 9)		5.1 ± 1.3^{bc} (n = 9)	4.6 ± 1.5^{bc} (n = 9)
II	150			5.2 ± 1.8^{bc} (n = 9)		3.7 ± 1^{c} (n = 8)	4 ± 1.1^{bc} (n = 7)
II	200			4.5 ± 1.6^{bc} (n = 8)		3.4 ± 1.3^{c} (n = 9)	3.6 ± 1^{c} (n = 5)
III	100			12 ± 3^{d} (n = 9)			10.3 ± 2.7^{d} (n = 9)
III	250			3 ± 1.3^{e} (n = 8)			♣
III	350				♣		
III	500			♣			

Mean values ± standard deviation. Different superscript letters indicate significant differences between results of their respective experiments. ^ = Approximate values. ♣ = No value due to dead postlarvae which did not survive the stress of the treatments. Values with common superscript letters were not significantly different at 95% confidence intervals.

However, the results of the postlarvae in experiment III (extremely high alkalinity and high total hardness) showed that the postlarvae were growing at a higher growth rate than those postlarvae reared in the low alkalinity and total hardness concentrations.

There were no significant differences ($P > 0.05$) among the growth rates of the postlarvae of the experiment I, regardless of alkalinity and total hardness concentrations. However, in experiment II, there was a high variation in the growth rates of the postlarvae. Statistically significant differences were found in the second experiment concerning the alkalinity levels ($P < 0.05$), as well as the total hardness ($P < 0.05$). Therefore, not only high alkalinity had a negative effect on the postlarvae growth rate, but also the total hardness did as well. The results of this second experiment showed that when both alkalinity and total hardness levels were high and in combination (above 100 mg L^{-1} as $CaCO_3$), there was an increasing negative synergistic effect on the growth rate as both factors increased. The results of experiment III, showed a dramatic decrease in the growth rate, when the alkalinity levels increased above 200 mg L^{-1} as $CaCO_3$. That significant decrease ($P < 0.05$) is very much greater when compared with the control treatment, but when compared with the results for experiment II of the growth rate of postlarvae at 1000 (mg L^{-1} as $CaCO_3$) total hardness and 200 (mg L^{-1} as $CaCO_3$) alkalinity, the growth rates are very similar. The postlarvae of the treatments with extremely high alkalinity 250 did not survive to the test, showing a strong negative effect of the alkalinity concentrations above 200 mg L^{-1} as $CaCO_3$.

As mentioned in the methodology, three groups of postlarvae were used in the three different experiments. Each group showed a different growth rate even when the postlarvae were held in very similar conditions in the control treatments (100 mg L^{-1} as $CaCO_3$ in both alkalinity and total hardness), therefore a transformation into percentage of the control group was done to compare the differences among the postlarvae. The results of that transformation into percentages are shown in Figure 7. With this transformation it was easy to see that the concentrations of alkalinity and total hardness higher than 100 mg L^{-1} as $CaCO_3$ reduced the growth rate of the postlarvae. This growth rate was significantly ($P < 0.05$) reduced when high alkalinity was in combination with high total hardness (i.e. 150 and 500 respectively). It was also seen from this transformation into percentages that the best growth rates were achieved at alkalinity levels of 25 mg L^{-1} as $CaCO_3$ and in combination with total hardness levels between 25 and 100 mg L^{-1} as $CaCO_3$. However, that was in percentages of the control groups, but in absolute terms the best growth rate was achieved at levels of 100 (mg L^{-1} as $CaCO_3$) for both alkalinity and total hardness.

When the alkalinity levels were lower than 100 mg L^{-1} as $CaCO_3$, it was shown that the growth rate had no trend at all regarding the increase of total hardness from 25 to 100 mg L^{-1} (as $CaCO_3$). When the alkalinity levels increased more than 100 mg L^{-1} (as $CaCO_3$) then, the total hardness had a negative effect on growth as it was also increased above 100 mg L^{-1} (as $CaCO_3$), although a high growth rate was obtained from the third experiment at levels of total hardness as high as 1000 mg L^{-1} as $CaCO_3$. A similar negative

response is shown when the total hardness level was above 100 mg L^{-1} (as $CaCO_3$) and the level of alkalinity increased from 100 to 200 mg L^{-1} (as $CaCO_3$). Furthermore, when the alkalinity levels increased above 250 the organisms just died within the first week of the experiment. Except for the treatment of 250 alkalinity and 1000 total hardness, in which the postlarvae survived until the third experimental moult.

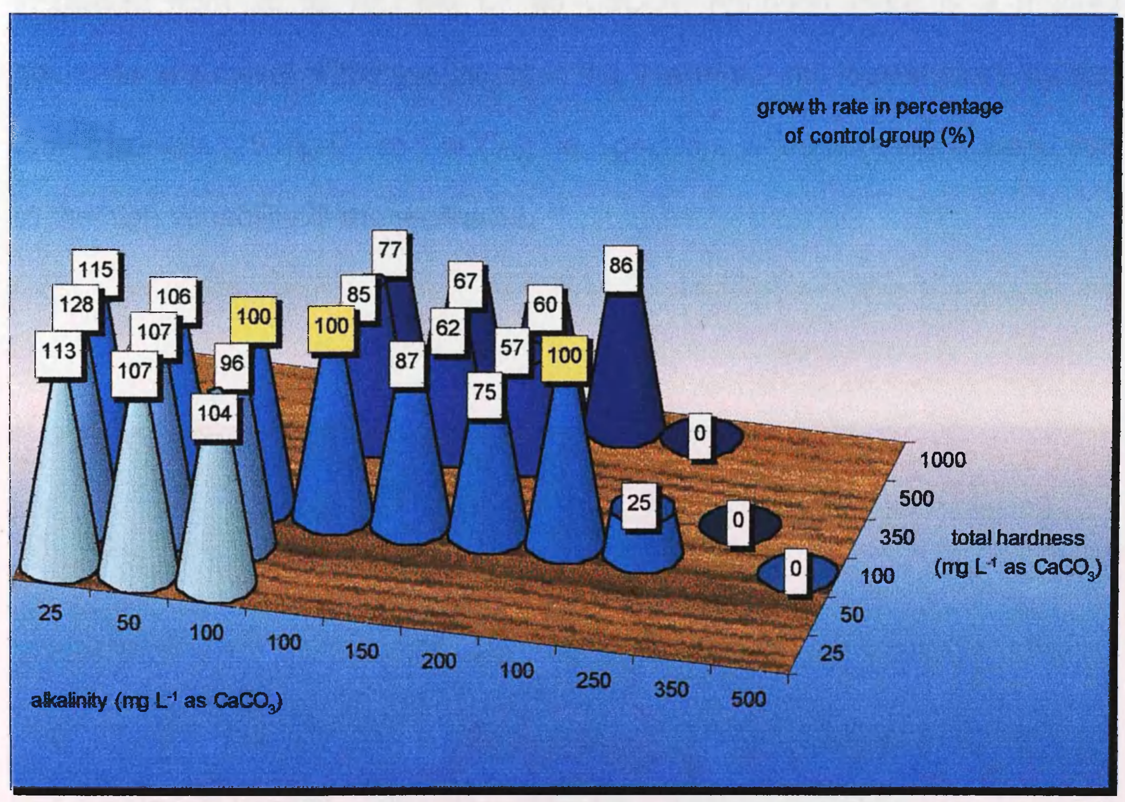


Figure 7. Growth rate of postlarvae of *M. rosenbergii* (De Man) represented in percentage of the control groups, which had 100 mg L^{-1} as $CaCO_3$ for both alkalinity and total hardness.

3.3.3. Survival

The survival of the three experimental postlarvae groups can be seen in Figure 8. There was a high variability in the survival of the postlarvae of the

first experiment, but statistically there were no significant differences regarding the alkalinity levels ($P > 0.05$) nor total hardness ($P > 0.05$) or their combined effect ($P > 0.05$). However, it can be seen that with total hardness levels of 50 mg L^{-1} as $CaCO_3$, their survival was stabilised at 88.9%, regardless of the alkalinity levels. Meanwhile, when the alkalinity was 50 mg L^{-1} as $CaCO_3$ their survival tended to decrease from 100 to 66.7% as the total hardness level increased from 25 to 100 mg L^{-1} as $CaCO_3$. Although there is a marked decrease in survival of the postlarvae in the treatment with lowest alkalinity and total hardness (25 mg L^{-1} as $CaCO_3$), no significant differences were found due to the high variability of the replicates.

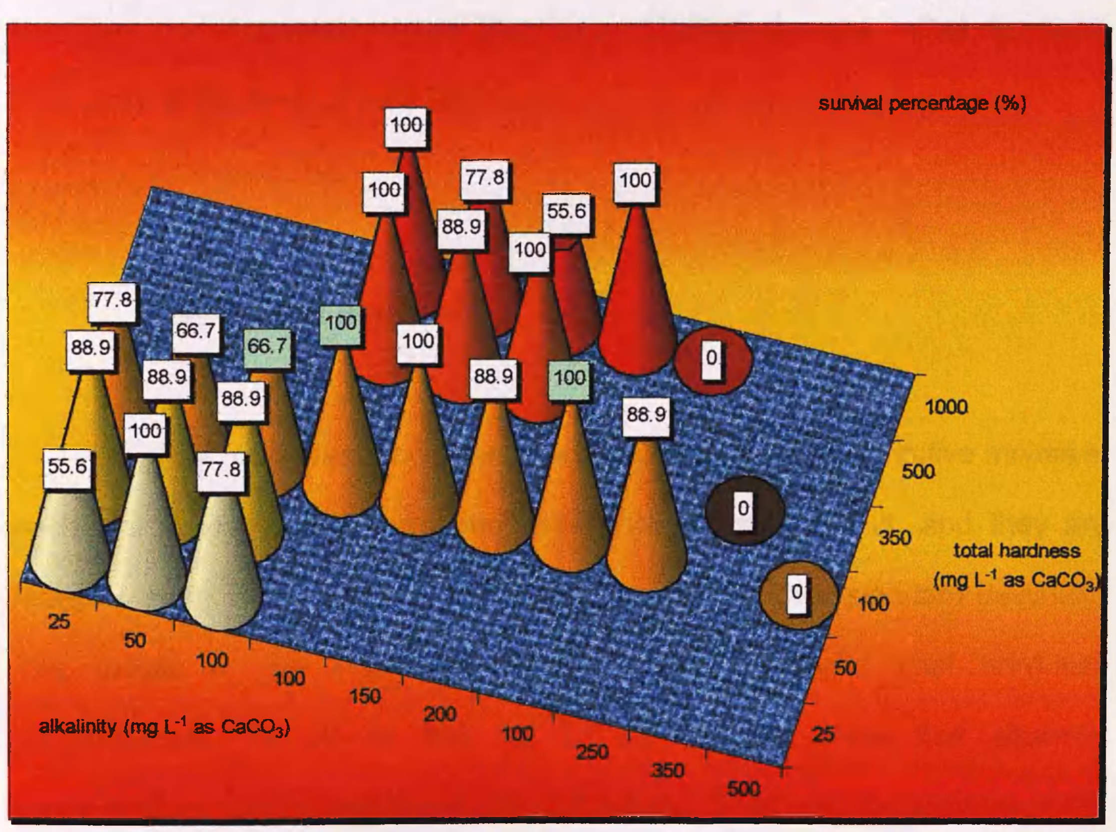


Figure 8. Survival of the postlarvae of *M. rosenbergii*.

Significant differences ($P < 0.05$) were found for the survival of the postlarvae of the second experiment, where the levels of alkalinity and total hardness were from moderate to high (100 to 200 (alkalinity) and 100 to 1000 (total hardness) in mg L^{-1} as $CaCO_3$). In experiment II, a declining gradient in the survival of the organisms is shown in Figure 8., where better survival was obtained when the levels of both factors (alkalinity and total hardness) were moderate (100 mg L^{-1} as $CaCO_3$) than when they were high i.e. 200 to 1000 mg L^{-1} as $CaCO_3$ for alkalinity and total hardness respectively. Indeed, the lowest survival for this experiment was found when the alkalinity was 200 mg L^{-1} as $CaCO_3$ and in combination with the highest total hardness (1000 mg L^{-1} as $CaCO_3$). The third experiment showed that levels of alkalinity of 250 mg L^{-1} as $CaCO_3$ in combination with high total hardness were lethal for the postlarvae and that any increment above that level of alkalinity was lethal for them regardless of the level of total hardness.

3.3.4. Moult frequency

The moult frequency was measured during five consecutive moults in each one of the experiments after one acclimatisation moult, and they are represented in Table 12. as mean values plus and minus a standard deviation. The results of the first experiment (low alkalinity and total hardness concentrations, i.e. 25 to 100 mg L^{-1} as $CaCO_3$), show that alkalinity concentrations had no significant effect ($P > 0.05$) on the moult frequency of the postlarvae. Meanwhile a significant increase ($P < 0.05$) of the moulting

frequency of the postlarvae was produced as the total hardness increased from 25 to 100 mg L^{-1} as $CaCO_3$.

Table 12. Moulting frequency (in days) of postlarvae of *M. rosenbergii*.

Experiment	^ Alkalinity (mg L^{-1} as $CaCO_3$)	^ Total hardness (mg L^{-1} as $CaCO_3$)					
		25	50	100	350	500	1000
I	25	7.6 ± 0.2^{a} (n = 5)	7.2 ± 0.5^{ab} (n = 8)	7.1 ± 0.4^{ab} (n = 7)			
	50	7.5 ± 0.5^{a} (n = 9)	6.9 ± 0.2^{ab} (n = 8)	6.7 ± 0.3^{b} (n = 6)			
	100	7.3 ± 0.3^{ab} (n = 7)	7.2 ± 0.5^{ab} (n = 8)	6.5 ± 0.4^{b} (n = 6)			
II	100			5.9 ± 0.6^{c} (n = 9)		5.9 ± 0.7^{c} (n = 9)	5.9 ± 0.7^{c} (n = 9)
	150			6 ± 0.7^{c} (n = 9)		5.4 ± 0.5^{c} (n = 8)	5.8 ± 0.5^{c} (n = 7)
	200			6 ± 0.5^{c} (n = 8)		5.7 ± 0.6^{c} (n = 9)	5.6 ± 0.7^{c} (n = 5)
III	100			6.4 ± 0.6^{d} (n = 9)			6.3 ± 0.3^{d} (n = 9)
	250			5.8 ± 0.2^{d} (n = 8)			♣
	350				♣		
	500			♣			

Mean values ± standard deviation. Different superscript letters indicate significant differences between results of their respective experiments. ^ = Approximate values. ♣ = No value due to dead postlarvae which did not survive due to the stress of the treatments. Values with common superscript letters were not significantly different at 95% confidence intervals.

The moult frequency did not have a wide variation between the treatments of the second experiment, and no significant differences were found ($P > 0.05$). However, there was a similar trend to increase the moulting frequency as both factors increased (alkalinity and total hardness). Meanwhile it was not possible to make a comparison between the six different treatments of the third experiment, in which three of the six experimental trials had no data due to a 100% mortality found by the end of the experiment in each of them.

However, there were no significant differences ($P > 0.05$) between the three different treatments in which the postlarvae survived the stress.

The moult frequency was transformed into percentages of the control treatments (Figure 9.) in order to see the general trend of the three experiments. This was a decrease of the moulting time as both alkalinity and total hardness increased. It can be seen that although no significant differences were found at low levels of alkalinity in the first experiment, the moult frequency tended to increase as the alkalinity increased at total hardness levels of 100 mg L^{-1} as $CaCO_3$.

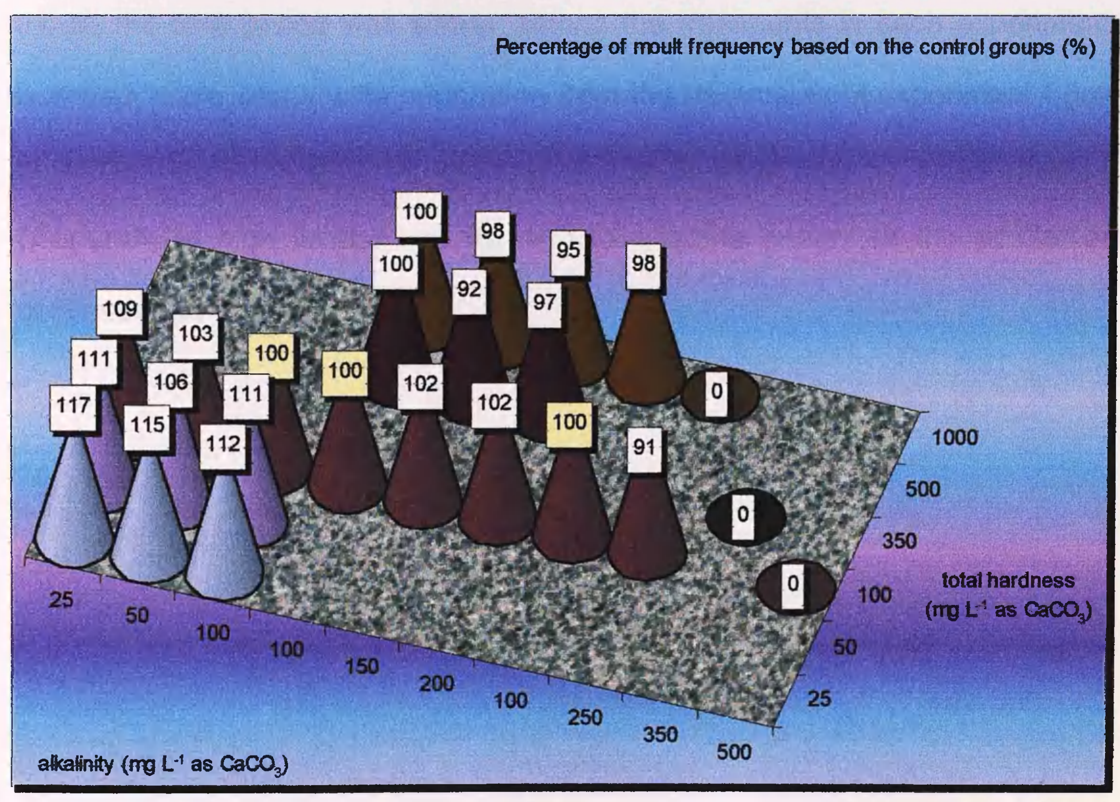


Figure 9. Moulting frequency of postlarvae of *M. rosenbergii*, represented in percentage of the control treatments.

The same trend was observed when the total hardness increased in all three alkalinity levels (25 to 100 mg L^{-1} as $CaCO_3$). In experiment II and III a similar trend is shown after conversion into percentages, where the postlarvae moulted more often as the total hardness and alkalinity increased. Figure 9. Moulting frequency of postlarvae of *M. rosenbergii*, represented in percentage of the control treatments.

Overall, the three groups of postlarvae responded in different ways, even when the control groups were in the same environmental condition. In Figure 10., which shows only representative values for each experiment and no standard deviation bars are included for more clarity, it is seen that in the first moult, the time period was similar among the treatments in each experiment, however, there was a wide separation from the treatments of experiment I (low to moderate levels of both alkalinity and total hardness) to experiments II (moderate to high levels) and III (high to extreme levels) as the postlarvae continued to moult.

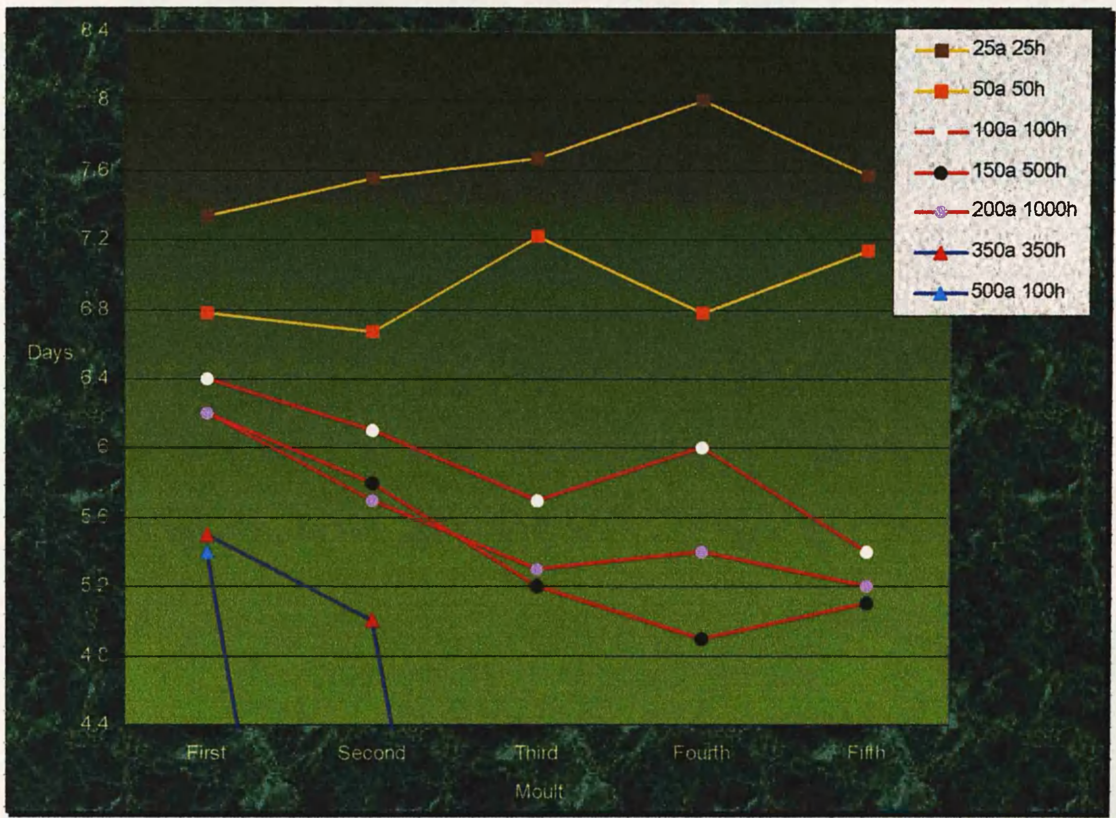


Figure 10. Mean values for a sequential moulting frequency of postlarvae *of M. rosenbergii* during the five experimental moults.
The letters "a" and "h" represent the alkalinity and total hardness concentrations in mg L^{-1} as $CaCO_3$ respectively. The yellow, red and blue lines represent treatments of experiment I, II and III respectively.

3.3.5. Carapace mineralization

3.3.5.1. Length-specific dry weight of the carapace

There were no significant differences (P > 0.5) of the length-specific dry weights of either the cast or the intermoult carapaces of the postlarvae reared in experiment I (Table 13.).

Table 13. Length-specific dry weight of the carapace of postlarvae of *M. rosenbergii* in mg mm^{-1}.

Carapace	Experiment	^ Alkalinity	^ Total hardness					
			25	50	100	350	500	1000
Cast	I	25	0.190 ± 0.059^{a} (n = 5)	0.183 ± 0.059^{a} (n = 8)	0.188 ± 0.035^{a} (n = 7)			
		50	0.202 ± 0.044^{a} (n = 9)	0.147 ± 0.056^{a} (n = 8)	0.195 ± 0.051^{a} (n = 6)			
		100	0.162 ± 0.064^{a} (n = 7)	0.197 ± 0.061^{a} (n = 8)	0.266 ± 0.087^{a} (n = 6)			
	II	100			0.174 ± 0.060^{bc} (n = 9)		0.207 ± 0.104^{bc} (n = 9)	0.162 ± 0.041^{bc} (n = 9)
		150			0.205 ± 0.045^{bc} (n = 9)		0.130 ± 0.027^{b} (n = 8)	0.251 ± 0.096^{c} (n = 7)
		200			0.225 ± 0.065^{bc} (n = 8)		0.183 ± 0.050^{bc} (n = 9)	0.138 ± 0.029^{bc} (n = 5)
	III	100			0.314 ± 0.079^{d} (n = 9)			0.275 ± 0.052^{de} (n = 9)
		250			0.216 ± 0.073^{e} (n = 8)			♣
		350				♣		
		500			♣			
Intermoult	I	25	0.903 ± 0.287^{f} (n = 5)	0.860 ± 0.163^{f} (n = 8)	0.684 ± 0.167^{f} (n = 7)			
		50	0.867 ± 0.167^{f} (n = 9)	0.870 ± 0.252^{f} (n = 8)	0.749 ± 0.116^{f} (n = 6)			
		100	0.885 ± 0.042^{f} (n = 7)	0.889 ± 0.207^{f} (n = 8)	0.963 ± 0.209^{f} (n = 6)			
	II	100			0.526 ± 0.112^{g} (n = 9)		0.463 ± 0.086^{g} (n = 9)	0.611 ± 0.211^{g} (n = 9)
		150			0.482 ± 0.163^{g} (n = 9)		0.364 ± 0.123^{g} (n = 8)	0.490 ± 0.115^{g} (n = 7)
		200			0.507 ± 0.233^{g} (n = 8)		0.444 ± 0.174^{g} (n = 9)	0.499 ± 0.202^{g} (n = 5)
	III	100			1.032 ± 0.277^{h} (n = 9)			0.993 ± 0.199^{h} (n = 9)
		250			0.412 ± 0.167^{f} (n = 8)			♣
		350				♣		
		500			♣			

Mean values ± standard deviation. Different superscript letters indicate significant differences between results of their respective experiments at 95% confidence intervals. ^ = Approximate values in mg L^{-1} as $CaCO_3$. ♣ = No value due to dead postlarvae which did not survive due to the stress of the treatments.

The second experiment (Table 13.) did not show any difference ($P > 0.05$) in either cast and intermoult carapaces regarding both alkalinity and total hardness. Although there was a significant interaction effect ($P < 0.05$) in the cast carapaces, during this second experiment, no trend was observed in the results. However, a negative effect ($P < 0.05$) was found in experiment III (Table 13.), when the alkalinity reached levels of 250 mg L^{-1} as $CaCO_3$, decreasing the

length-specific dry weight in both cast and intermoult carapaces. Intermoult carapaces were always heavier than cast carapaces in the three different experiments.

3.3.5.2. Calcium content in the carapaces

The calcium content of the carapaces in the three experiments is shown in Table 14. Neither alkalinity nor total hardness had a significant effect ($P > 0.05$) on the calcium content of both cast and intermoult carapaces of the postlarvae in experiment I. However, in experiment II both factors affected ($P < 0.05$) the intermoult carapaces, which contained more calcium as the total hardness increased at level of alkalinity between 100 and 150, but the trend was inverse when the level of alkalinity increased up to 200 mg L^{-1} as $CaCO_3$. The cast carapaces were not significantly affected ($P > 0.05$). Statistically no significant differences were found for both cast and intermoult carapaces in the third experiment, however a marked trend was found to reduce the calcium content in both cast and intermoult carapaces as the alkalinity increased. The results in Table 14., shown that cast carapaces have more calcium per gram of carapace than intermoult ones.

Table 14. Calcium content in the carapace of postlarvae of *M. rosenbergii* in mg g^{-1}.

Carapace	Experiment	^ Alkalinity	^ Total hardness					
			25	50	100	350	500	1000
Cast	I	25	202.6 ± 58.7^{a} (n = 5)	297.9 ± 102.5^{a} (n = 8)	240.6 ± 69.7^{a} (n = 7)			
		50	271.9 ± 71.7^{a} (n = 9)	335.6 ± 160.3^{a} (n = 8)	345.9 ± 78^{a} (n = 6)			
		100	341.9 ± 103.1^{a} (n = 7)	345.9 ± 171.5^{a} (n = 8)	273.4 ± 43.3^{a} (n = 6)			
	II	100			219.8 ± 63^{b} (n = 9)		243.2 ± 43.8^{b} (n = 9)	207.8 ± 35.9^{b} (n = 9)
		150			215.1 ± 30.5^{b} (n = 9)		254.4 ± 52^{b} (n = 8)	243.0 ± 64.4^{b} (n = 7)
		200			224.0 ± 15.8^{b} (n = 8)		241.9 ± 57.7^{b} (n = 9)	214.0 ± 26.1^{b} (n = 5)
	III	100			343.8 ± 41.8^{c} (n = 9)			347.6 ± 47.9^{c} (n = 9)
		250			319.3 ± 71.4^{c} (n = 8)			♣
		350				♣		
		500			♣			
Intermoult	I	25	188.8 ± 29.5^{d} (n = 5)	221.4 ± 37.3^{d} (n = 8)	199.4 ± 41.0^{d} (n = 7)			
		50	202.2 ± 35.2^{d} (n = 9)	214.2 ± 40.1^{d} (n = 8)	204.9 ± 39.9^{d} (n = 6)			
		100	208.6 ± 26.2^{d} (n = 7)	196.1 ± 30.3^{d} (n = 8)	206.7 ± 27.9^{d} (n = 6)			
	II	100			145.2 ± 17.9^{e} (n = 9)		171.9 ± 16.6^{efg} (n = 9)	183.8 ± 24.6^{g} (n = 9)
		150			150.3 ± 24.7^{ef} (n = 9)		150.2 ± 19.2^{efg} (n = 8)	172.0 ± 11.3^{efg} (n = 7)
		200			178.5 ± 23.7^{efg} (n = 8)		179.5 ± 23.3^{fg} (n = 9)	177.2 ± 23.5^{efg} (n = 5)
	III	100			230.3 ± 31.4^{h} (n = 9)			215.3 ± 19.2^{h} (n = 9)
		250			206.7 ± 24.9^{h} (n = 8)			♣
		350				♣		
		500			♣			

Mean values ± standard deviation. Different superscript letters indicate significant differences between results of their respective experiments at 95% confidence intervals. ^ = Approximate values in mg L^{-1} as $CaCO_3$. ♣ = No value due to dead postlarvae which did not survive due to the stress of the treatments.

3.3.5.3. Length-specific calcium content

The length-specific calcium content in the carapaces is inverse to the calcium content of the carapace since the intermoult carapace had a higher length-specific calcium content than the cast carapace in the three different experiments (Table 15.).

Table 15. Length-specific calcium content in the carapace of postlarvae of *M. rosenbergii* in mg mm^{-1}.

Carapace	Experiment	^ Alkalinity	^ Total hardness					
			25	50	100	350	500	1000
Cast	I	25	0.034 ± 0.014^{a} (n = 5)	0.050 ± 0.006^{ab} (n = 8)	0.046 ± 0.016^{ab} (n = 7)			
		50	0.053 ± 0.011^{ab} (n = 9)	0.043 ± 0.013^{ab} (n = 8)	0.064 ± 0.020^{ab} (n = 6)			
		100	0.053 ± 0.026^{ab} (n = 7)	0.059 ± 0.015^{ab} (n = 8)	0.071 ± 0.019^{b} (n = 6)			
	II	100			0.037 ± 0.014^{cd} (n = 9)		0.048 ± 0.020^{cd} (n = 9)	0.033 ± 0.008^{c} (n = 9)
		150			0.045 ± 0.014^{cd} (n = 9)		0.032 ± 0.004^{c} (n = 8)	0.057 ± 0.017^{d} (n = 7)
		200			0.051 ± 0.015^{cd} (n = 8)		0.043 ± 0.011^{cd} (n = 9)	0.030 ± 0.009^{c} (n = 5)
	III	100			0.103 ± 0.025^{e} (n = 9)			0.095 ± 0.018^{ef} (n = 9)
		250			0.072 ± 0.023^{f} (n = 8)			♣
		350				♣		
		500			♣			
Intermoult	I	25	0.165 ± 0.040^{g} (n = 5)	0.187 ± 0.032^{g} (n = 8)	0.133 ± 0.032^{g} (n = 7)			
		50	0.177 ± 0.051^{g} (n = 9)	0.185 ± 0.056^{g} (n = 8)	0.152 ± 0.021^{g} (n = 6)			
		100	0.188 ± 0.025^{g} (n = 7)	0.171 ± 0.033^{g} (n = 8)	0.197 ± 0.036^{g} (n = 6)			
	II	100			0.078 ± 0.025^{h} (n = 9)		0.079 ± 0.015^{h} (n = 9)	0.114 ± 0.048^{h} (n = 9)
		150			0.071 ± 0.021^{h} (n = 9)		0.055 ± 0.022^{h} (n = 8)	0.084 ± 0.017^{h} (n = 7)
		200			0.093 ± 0.050^{h} (n = 8)		0.081 ± 0.037^{h} (n = 9)	0.088 ± 0.033^{h} (n = 5)
	III	100			0.231 ± 0.056^{i} (n = 9)			0.194 ± 0.019^{i} (n = 9)
		250			0.082 ± 0.031^{j} (n = 8)			♣
		350				♣		
		500			♣			

Mean values ± standard deviation. Different superscript letters indicate significant differences between results of their respective experiments at 95% confidence intervals. ^ = Approximate values in mg L^{-1} as $CaCO_3$. ♣ = No value due to dead postlarvae which did not survive due to the stress of the treatments.

Neither alkalinity nor total hardness had a significant effect ($P > 0.05$) on the length-specific calcium content of intermoult carapaces of the postlarvae reared in experiment I. However, alkalinity significantly influenced ($P < 0.05$) the length-specific calcium content of the cast carapaces, where calcium content increased as alkalinity increased, and a similar trend was observed when the total hardness was increased. Neither the alkalinity nor the total hardness had a

significant effect on the length-specific calcium content of the cast carapaces in experiment II ($P > 0.05$). However, there was a significant interaction, reducing the calcium content at levels of 150 alkalinity and 500 total hardness and 200 alkalinity and 1000 total hardness ($P < 0.05$). No significant differences were found for the intermoult carapaces of experiment II. However, in experiment III, both cast and intermoult carapaces showed significant differences ($P < 0.05$), where the increase in alkalinity induced a decrease in the length-specific calcium content.

3.3.5.4. Magnesium content in the carapaces

Magnesium content for cast and intermoult carapaces of the three experiments are shown in Table 16. Alkalinity, total hardness or their interaction had no significant effect ($P > 0.05$) on the magnesium content in the cast and intermoult carapaces of the postlarvae during the first experiment. However, as total hardness was increased, there was a significant ($P < 0.05$) increase in magnesium content in both cast and intermoult carapaces of experiment II. The results of experiment III showed that both factors (alkalinity and total hardness) influenced the content of magnesium in both the cast and intermoult carapaces, following the same trend as in experiment II, where an increase in magnesium content was related to an increase in both alkalinity and total hardness. The magnesium content of cast carapaces was higher than the one in the intermoult carapaces for the three experimental groups of postlarvae.

Table 16. Magnesium content in the carapaces of postlarvae of *M. rosenbergii* in mg g^{-1}.

Carapace	Experiment	^ Alkalinity	^ Total hardness					
			25	50	100	350	500	1000
Cast	I	25	18.1 ± 8.9^{a} (n = 5)	25.2 ± 15.6^{a} (n = 8)	22.9 ± 8.8^{a} (n = 7)			
		50	27.8 ± 12.4^{a} (n = 9)	31.4 ± 16.4^{a} (n = 8)	23.4 ± 6.9^{a} (n = 6)			
		100	27.8 ± 14^{a} (n = 7)	21.8 ± 13.2^{a} (n = 8)	18.7 ± 14.1^{a} (n = 6)			
	II	100			10.7 ± 3.3^{bc} (n = 9)		10.7 ± 4.6^{bc} (n = 9)	15.3 ± 5^{c} (n = 9)
		150			7.7 ± 2.7^{b} (n = 9)		13.1 ± 5.1^{bc} (n = 8)	11.6 ± 3.2^{bc} (n = 7)
		200			6.6 ± 1.8^{b} (n = 8)		12.0 ± 6^{bc} (n = 9)	13.0 ± 2.5^{bc} (n = 5)
	III	100			14.1 ± 2.6^{d} (n = 9)			21.0 ± 5.7^{de} (n = 9)
		250			22.2 ± 8.2^{e} (n = 8)			♣
		350				♣		
		500			♣			
Intermoult	I	25	4.9 ± 2.2^{f} (n = 5)	6.1 ± 2.8^{f} (n = 8)	8.1 ± 3.1^{f} (n = 7)			
		50	6.5 ± 1.6^{f} (n = 9)	6.7 ± 3.7^{f} (n = 8)	10.9 ± 9.1^{f} (n = 6)			
		100	7.3 ± 1.9^{f} (n = 7)	5.9 ± 1.9^{f} (n = 8)	5.7 ± 1.6^{f} (n = 6)			
	II	100			4.5 ± 0.9^{g} (n = 9)		6.0 ± 1^{gh} (n = 9)	7.0 ± 1.5^{h} (n = 9)
		150			4.8 ± 1.1^{g} (n = 9)		6.2 ± 0.6^{gh} (n = 8)	6.4 ± 2.7^{gh} (n = 7)
		200			4.7 ± 1.3^{g} (n = 8)		6.0 ± 0.5^{gh} (n = 9)	7.4 ± 1.7^{h} (n = 5)
	III	100			5.3 ± 0.9^{i} (n = 9)			8.2 ± 1.1^{k} (n = 9)
		250			16.9 ± 3.2^{j} (n = 8)			♣
		350				♣		
		500			♣			

Mean values ± standard deviation. Different superscript letters indicate significant differences between results of their respective experiments at 95% confidence intervals. ^ = Approximate values mg L^{-1} as $CaCO_3$. ♣ = No value due to dead postlarvae which did not survive due to the stress of the treatments.

3.3.5.5. Length-specific magnesium content

The results of experiment I shown that there were no significant differences in the length-specific magnesium content of either cast or intermoult carapaces (Table 17.), neither was there a trend regarding the alkalinity, total hardness or their interaction. There were no significant differences regarding the levels of alkalinity ($P > 0.05$), but total hardness did produced significant

differences (P < 0.05) for both cast and intermoult carapaces of experiment II (Table 17.).

Table 17. Length-specific magnesium content in the carapace of postlarvae of *M. rosenbergii* in mg mm^{-1}.

Carapace	Experiment	^ Alkalinity	^ Total hardness					
			25	50	100	350	500	1000
Cast	I	25	0.003 ± 0.001^{a} (n = 5)	0.004 ± 0.001^{a} (n = 8)	0.004 ± 0.001^{a} (n = 7)			
		50	0.005 ± 0.001^{a} (n = 9)	0.004 ± 0.001^{a} (n = 8)	0.004 ± 0.001^{a} (n = 6)			
		100	0.004 ± 0.001^{a} (n = 7)	0.004 ± 0.001^{a} (n = 8)	0.004 ± 0.001^{a} (n = 6)			
	II	100			0.002 ± 0.001^{bc} (n = 9)		0.002 ± 0.0^{bc} (n = 9)	0.002 ± 0.001^{bc} (n = 9)
		150			0.002 ± 0.0^{b} (n = 9)		0.002 ± 0.0^{bc} (n = 8)	0.003 ± 0.001^{c} (n = 7)
		200			0.001 ± 0.0^{b} (n = 8)		0.002 ± 0.001^{bc} (n = 9)	0.002 ± 0.00^{bc} (n = 5)
	III	100			0.004 ± 0.001^{d} (n = 9)			0.006 ± 0.001^{e} (n = 9)
		250			0.004 ± 0.0^{d} (n = 8)			♣
		350				♣		
		500			♣			
Intermoult	I	25	0.004 ± 0.001^{f} (n = 5)	0.005 ± 0.001^{f} (n = 8)	0.005 ± 0.001^{f} (n = 7)			
		50	0.006 ± 0.001^{f} (n = 9)	0.005 ± 0.001^{f} (n = 8)	0.004 ± 0.001^{f} (n = 6)			
		100	0.006 ± 0.002^{f} (n = 7)	0.005 ± 0.001^{f} (n = 8)	0.005 ± 0.002^{f} (n = 6)			
	II	100			0.002 ± 0.0^{g} (n = 9)		0.003 ± 0.001^{gh} (n = 9)	0.004 ± 0.001^{h} (n = 9)
		150			0.002 ± 0.001^{g} (n = 9)		0.002 ± 0.001^{g} (n = 8)	0.003 ± 0.001^{gh} (n = 7)
		200			0.002 ± 0.001^{g} (n = 8)		0.003 ± 0.001^{gh} (n = 9)	0.004 ± 0.001^{gh} (n = 5)
	III	100			0.006 ± 0.001^{i} (n = 9)			0.008 ± 0.002^{j} (n = 9)
		250			0.006 ± 0.002^{ij} (n = 8)			♣
		350				♣		
		500			♣			

Mean values ± standard deviation. Different superscript letters indicate significant differences between results of their respective experiments at 95% confidence intervals. ^ = Approximate values mg L^{-1} as $CaCO_3$. ♣ = No value due to dead postlarvae which did not survive due to the stress of the treatments.

In experiment III there were significant differences ($P < 0.05$) in both cast and intermoult carapaces, where the cast carapaces had more magnesium as the total hardness increased, but not the intermoult ones (Table 17.). Although the amounts of magnesium were relatively small, the intermoult carapaces had slightly more length-specific magnesium content than the cast ones.

3.4. DISCUSSION

3.4.1. Alkalinity and total hardness in the rearing tanks

The obtained and required alkalinity and total hardness were not always the same (see section 3.3.1). This variation in the levels of both factors was caused by the addition of the chemicals and water in high amounts (manually) to the system in each treatment needed, since the system was a flow through water system. It was very difficult to maintain the required levels for both alkalinity and total hardness when the levels of alkalinity were required to be above 200 and the total hardness as high as 1000 mg L^{-1} as $CaCO_3$, however, those variations of the concentrations in the media were minor when the levels of alkalinity were under 200 mg L^{-1} as $CaCO_3$. The titration results for each treatment were very accurate and not much variation was recorded during the preparation of the water media used in the trials previous to the actual experiments.

Wurts (1993) mentioned that calcium would precipitate or come out of solution as an insoluble carbonate if the pH is 8.3 or higher. In this study, most of the pH measurements were above 8.3 when the alkalinity levels where 100 mg L^{-1} as $CaCO_3$ or above, and reaching up to 9 in the pH scale when the alkalinity was as high as 350 mg L^{-1} as $CaCO_3$. However, a precipitate of calcium carbonate started to appear on the walls of the header tanks and inside of the tubing of the system in the third experiment with extreme alkalinity concentrations (up to 500 mg L^{-1} as $CaCO_3$), after a couple of days. Only in that experiment III was it difficult to maintain the required concentrations of carbonate and calcium in the water media, e.g. the concentrations were down to mean values of 266.6 and 851 mg L^{-1} as $CaCO_3$ when the alkalinity and total hardness were required to be 500 and 1000 mg L^{-1} as $CaCO_3$ respectively. Carbon dioxide (CO_2) was a possible factor causing the differences in the precipitation times between the trials and the actual experiment (Hogg *et al.*, 1957; Zumdahl, 1993; McQueen, E., personal communications, 1998). Dissolved carbon dioxide in the water media was exposed to the relatively large surface of the water of the header tank in the interface water-atmosphere, and therefore any escape into the air reduced the acidity of the water and forced the excess carbonates to produce insoluble calcium and magnesium carbonates once the saturation levels of the water media were reached.

Most of the species of the genus *Macrobrachium* that live in Malaysian waters, extend their habitats from acidic (pH < 6) to hard waters (pH > 8) (Johnson, 1967). Smith *et al.*, reported in 1976 that *Macrobrachium* spp. can grow under poor conditions i.e. low pH (5.5), low water hardness (5 mg L^{-1})

and low dissolved oxygen concentration (1.7 mg L^{-1}). Furthermore, Zhou (1987) and Brown *et al.*, (1991) found that *M. rosenbergii* postlarvae grow better at total hardness concentrations lower than 40 and 53 mg L^{-1} as $CaCO_3$ respectively. That suggests that *M. rosenbergii* has no problems with living in low water hardness and alkalinity, and that is why lower levels of these two parameters were not studied in this work. In any case, the actual interest for expanding the culture of this species is mainly in countries that show problems of having very hard or alkaline water sources and not soft waters.

3.4.2. Growth rate

After the results of the first two experiments, the trend of the growth rate was to decrease as the alkalinity increased, in order to conclude on this, another experiment was done increasing the alkalinity to extreme levels and maintaining the same management and environmental conditions. The results of the third experiment shown that indeed alkalinity over 200 mg L^{-1} as $CaCO_3$ sharply reduced the growth rate, and the effect was more severe when there was a combination with high total hardness (1000 mg L^{-1} as $CaCO_3$). This clearly indicated the effect of alkalinity and total hardness in the growth rate of the postlarvae of *M. rosenbergii.* However, other factors, which could possible affect the differences in the growth rate are discussed in this section.

Although there were control treatments in the experiments of this study, the growth rate of the postlarvae was quite different among them.

Similarly, when analysing growth rates of *M. rosenbergii* reported in other studies for organisms of similar initial sizes, it is shown that there is a great variation from one experimental group to the other (Table 18.). That makes it difficult to compare the results, furthermore, slight differences in the initial weight or the environmental conditions and the diet could accentuate that variation of their growth rate.

Table 18. Growth rate (mg day^{-1}) of *M. rosenbergii* at different levels of hardness and alkalinity (according to different authors).

^ Hardness (mg L^{-1} as $CaCO_3$)	^ Alkalinity (mg L^{-1} as $CaCO_3$)						Initial weight (g)	Reference
	10	25	100	100	250	**		
19						3.7	0.1 - 0.2	Brown *et al.*, (1991)
20	4.4	2.9	4	5.1	1.6		0.12 - 0.13	Latif (1992)
20	9.5	9.6	7	13.7	3.8		0.39 - 0.56	Latif (1992)
20	26						0.04	Vasquez *et al.*, (1989)
75						2.7	0.2 - 0.5	Brown *et al.*, (1991)
80	2.5		2.6	3	0.9		0.12 - 0.13	Latif (1992)
80	6		5.4	12.1	3.1		0.39 - 0.56	Latif (1992)
112	29						0.04	Vasquez *et al.*, (1989)
144						0.5	0.2 - 0.5	Brown *et al.*, (1991)
160	1.8		1.8	2.8	1.8		0.12 - 0.13	Latif (1992)
160	5.4		5.3	9.6	3.5		0.39 - 0.56	Latif (1992)
172						2.5	0.1 - 0.2	Brown *et al.*, (1991)
225	17						0.04	Vasquez *et al.*, (1989)
320				3			0.12 - 0.13	Latif (1992)
320				9.7			0.39 - 0.56	Latif (1992)
450	11						0.04	Vasquez *et al.*, (1989)
500		1.9					0.12 - 0.13	Latif (1992)
1000		2.5					0.12 - 0.13	Latif (1992)
1000		9.8					0.39 - 0.56	Latif (1992)

^ = Approximate concentration levels. ** = Alkalinity concentrations equal to hardness.

Johnson already mentioned in 1967 that variability between results obtained from laboratory organisms and wild organisms would be unavoidable, due to the inevitable differences between a laboratory and a natural environment.

The results of the growth rate of the organisms used in this work and shown in section 3.3.2., are on a similar scale as those reported by Brown *et al.*, (1991) and Latif (1992). However, an important aspect to notice from Table 18., is the high growth rate of the postlarvae obtained in the experiments by Vasquez *et al.*, (1989). Their experiments were carried out in tanks with added mud from the grow-out ponds. Furthermore the chemical composition of their experimental water was made up just by adding agricultural gypsum ($CaSO_4 \cdot 2H_2O$) to the water. Meanwhile, in the other two studies (Brown *et al.*, 1991; Latif, 1992) as well as in the present one, the water used was deionized and then the chemicals were added according to the HMSO formula in order to make up the required calcium and carbonate concentrations in the water column. Two possible reasons for the highest growth rate of the organisms used by Vasquez *et al.*, (1989) could be: A) trace elements and B) the social enhancement of growth.

A) In the study of Vasquez *et al.*, (1989), some trace elements necessary for the development of the species were included by the use of tap water plus the pond mud, which were not present in the other two studies of Table 18. and neither in the experimental water used in this study. For example, Porcella *et al.*, (1969) mentioned that the total

accumulation of strontium and calcium in *Daphnia magna* was related directly to the environmental concentrations of these ions, and indeed, Funge-Smith (1991) found that strontium dictates the magnesium regulation in adult *M. rosenbergii* during its moult cycle, and bromide is necessary for its metabolism in the larval and postlarval stages. He concludes that both bromide and strontium may be essential for the species. Therefore, if the preparation of the culture media was lacking some essential elements then, that could be a reason for the poor growth rate, however, small amounts of trace elements are provided by the food intake, but those amounts were not measured in this study.

B) Another reason for the increased growth rate in the study of Vasquez *et al.*, (1989) is the social enhancement of growth, since Ra'anan and Cohen (1985) found that communally held individuals of *M. rosenbergii* developed a faster growth rate than individually held organisms, and the experiments of Vasquez *et al.*, (1989) were carried out in communal groups of prawns.

Another factor apparently influencing the differences in the growth rate is the initial weight of the experimental prawns. Howlader and Turjoman (1984) reported a high hardness related increase in growth rate inhibition of *M. rosenbergii* as the prawns were bigger. Latif in 1992, found that heavier organisms (0.3 to 0.5 g) were growing better than the lighter ones (0.1 g). Similarly, in the present work, heavier organisms (0.18 g) were growing better than the lighter ones (0.14 g). However, when the organisms were of a similar

initial weight, (0.17 and 0.18 g) there were still differences in the growth rate of the postlarvae used in the present study. Similarly in the study of Brown *et al.*, (1991) lighter prawns were growing similarly or even better than heavier ones. Furthermore, Vasquez *et al.*, (1989) used organisms much lighter than the ones used in all the previous described works and they had a higher growth rate. Therefore, although the initial weight may have some influence in the initial development of the organisms under stressing conditions, it seems that it is not a major cause for the differences in growth rate. That suggestion is in agreement with the results obtained by Brown *et al.*, (1991) who combined all the experimental groups of postlarvae (different initial weights), and found a reduction in the moulting rate and an increase in growth of *M. rosenbergii* when the organisms were reared at low total hardness, regardless of their average initial weight.

Rearing space could be another factor affecting the growth rate. During this study, some postlarvae escaped before the start of the experimental trials and were living in the water surrounding the experimental trays, and it was noticed that those postlarvae although they were not fed properly, had a higher growth rate than most of the experimental postlarvae kept individually in the mesh pots. Aiken and Waddy (1978) found a formula for a non restricting growth holding area for lobsters, where 55 times the square of the carapace length (CL) should allow the lobsters to growth without space restrictions. However, Wickins and Beard (1991) found that size of the containers was not a restriction for the first instars of individually reared lobsters, but as soon as the lobsters reached a bigger size (> 60 mm carapace length) the size of the

container was reducing their growth and then the small organisms were able to catch up with the fast growing ones.

According to the findings of Aiken and Waddy (1978), an area of 31 cm^2 would be enough for an unrestricted growth of the organisms, taking into account that the average CL of the postlarvae in this experiments was 7.5 ± 1 mm. That area is one third of the actual area used to rear each individual postlarvae during this study. However, *M. rosenbergii* is a migratory species, especially from rivers and estuaries, which implies that the organisms have to move or swim constantly from one point of the river to another, depending on their life cycle stages and other external factors such as food availability and environmental changes. Therefore, it could be that *M. rosenbergii* requires more space for movement than lobsters, indicating that they require an increase in the "suitable" space for its enclosure during experimental trials, especially during stage "E" of the moulting cycle.

The differences in the growth rate of the experimental postlarvae of this study, were not caused by the effect of the diet used, since the diet was a good balance of nutrients (section 2.2.) and the level of protein (minimum of 39%) was much higher than the ones suggested by some authors. For instance, Gomez Diaz *et al.*, (1988) found that the required protein level was between 13 and 25% on the diet of *M. rosenbergii*. Garcia-Perez and Cortes-Cortes (1993) used diets with a 30% of protein in their experiments, obtaining growth rates up to 370 mg day^{-1} in pond culture of *M. rosenbergii*. Johnson (1982) mentioned that addition of high protein diets to the prawn culture can sometimes help to

increase growth and moult frequency and this can help to eliminate the effect of parasitic protozoa, which interfere with the feeding of the prawn. Furthermore, Murugadas and Pandian (1991) found that the higher the protein (> 35%), the better the development of *M. nobilii* including the ovarian maturation, whilst at 10% protein diets, their prawns succumbed before reaching maturity. They mentioned that the lower the dietary protein, the lower the growth and longer the time to attain the minimum body weight for maturity (600 mg). However, in 1982 Boonyaratpalin and New experimented with three diets containing different protein levels and found that there was no significant difference in the growth, production, survival and feed conversion of the freshwater prawn *M. rosenbergii* when reared under the three different diets. The levels of protein included in their diets were 15, 25 and 35%. They mentioned that from an economical point of view, this indicated that 15% inclusion is the best. Therefore malnutrition is not a reason for the results of these experiments.

Two important factors that affected the growth rate of the postlarvae are environmental total hardness and alkalinity, where the later carried an unavoidable increase in the pH as it was increased:

- Low levels of calcium carbonate where not affecting the growth of *M. rosenbergii* during the present study. Wurts (1993) mentioned that fish can lose calcium into the water when they are in low calcium waters, and then they have to use energy and ions supplied by their food to re-absorb lost calcium salts. That could be the case for extremely low calcium waters where prawns could lose their calcium through the exuviae, however, as mentioned

earlier, low hardness concentrations are favourable for the growth rate of *M. rosenbergii* (Cripps and Nakamura, 1979; Zhou 1987; Brown *et al.,* 1991). That suggests that prawns are well adapted to live in conditions with low water hardness where they are able to obtain their required calcium from the food that they may consume and therefore, the low environmental calcium is not a limiting factor for their development.

- However, high concentration of calcium carbonate affected the growth rate. Indicating a cross over effect of high hardness and alkalinity. In the study of Zhou (1987), he found that growth was higher as the hardness was kept lower and *vice versa*, and similar results were obtained by Brown *et al.*, (1991), who found that the growth rate declined at total hardness levels higher than 53 mg L^{-1} as $CaCO_3$. In those two cases, the levels of alkalinity were similarly high to the levels of total hardness. However, contrary to these reports where high environmental calcium was harmful for the development of *M. rosenbergii*, some studies like the one provided by Bartlett and Enkerlin in 1983, have found that *M. rosenbergii* is capable of regulating high environmental calcium (around 1000 mg L^{-1} as $CaCO_3$) as long as the alkalinity is low. Similarly, Sukadi (1989) found no differences in the growth rate of *M. rosenbergii* when he increased the environmental hardness with gypsum ($CaSO_4 \cdot 2H_2O$) from a well water supply from 18 to 300 mg L^{-1} as $CaCO_3$ and the alkalinity was maintained at 10 mg L^{-1} as $CaCO_3$. Howlader and Turjoman (1984) mentioned that a negative effect in the growth rate of *M. rosenbergii* was due to high hardness levels (688 to 987 mg L^{-1} as $CaCO_3$). However, the alkalinity levels in their study were relatively high (160

to 171 mg L^{-1} as $CaCO_3$) and a synergistic effect must have been influencing the results of the growth of their organisms. Indeed, alkalinity levels for the species in natural waters of Malaysia are between 4 and 78 mg L^{-1} as $CaCO_3$ with an average of 30.8 mg L^{-1} as $CaCO_3$ (Johnson, 1967). Thus confirming that high alkalinity alone or in combination with high hardness has a greater effect in the growth of *M. rosenbergii* than total hardness itself.

- Freshwater highly saturated with calcium carbonate, could affect the freshwater prawns in a similar way as salinity could affect marine crustaceans. Spaargaren (1975; 1976) mentioned that the consumption of energy in the shore crab *Carcinus maenas* was low when the ion concentration in the media was similar to the isosmotic point, and Stern *et al.*, in 1984, suggested that more energy is required when the ion gradient increases between the body fluids and external medium. The same has been suggested in general for a marine animal, where the least energy is expended if it is maintained in an isosmotic medium, due to the reduced energy expenditure on osmoregulation (Canagaratnam, 1959, cited in: Funge-Smith, 1991). So even if that is not the case for freshwater adult prawns (Singh, 1980), it may be for early postlarvae, which are newly transformed from being brackish water dependant larvae, to freshwater dependant postlarvae. In highly calcium carbonate enriched waters, the freshwater postlarvae could react as the marine animals in an hyperosmotic media, where they will have to spend a lot of energy to get rid of the excess ions and therefore the growth will be reduced or even stopped if the

organisms are using much of their metabolic energy to keep an optimal ionic balance.

- If the alkalinity increases, the pH will increase, both causing a synergistic effect on the development of *M. rosenbergii*. Johnson (1967) found a pH range of 5.2 to 6.2 with an average of 5.8 in some natural waters containing *M. rosenbergii*. Wickins (1984) mentions that high pH causes stress in crustaceans and reduces their growth rate and survival. In the present thesis the pH was high in the treatments with high alkalinity, that condition was an additional factor to produce stress for the prawns which were already using metabolic energy by trying to regulate their ionic requirements in high environmental concentrations of calcium, magnesium and carbonates.

3.4.3. Survival

There was no relationship between the trend of the growth rate of the postlarvae and their survival under any of the treatments of the first experiment. However, when the alkalinity increased in experiments II and III, there was a positive relationship between the poor growth and poor survival, especially when the levels of alkalinity were close to 200 mg L^{-1} as $CaCO_3$ and the levels of total hardness increased. From the results of experiment III it is clear that alkalinity levels above 200 mg L^{-1} as $CaCO_3$ had a lethal effect on the postlarvae of *M. rosenbergii*. Sukadi (1989) found a decrease in the survival of postlarvae of *M. rosenbergii* from 51.4 to 26% when the hardness increased

from 18 to 300 mg L^{-1} as $CaCO_3$. However, in the study of Vasquez *et al.*, (1989) there was inhibition of the postlarval growth rate, but no negative effects were shown in the survival. That could be related to the low alkalinity level that they used in their experiments (10 mg L^{-1} as $CaCO_3$). Zhou (1987) studied the response of *M. rosenbergii* at total hardness varying from 40 to 320 mg L^{-1} as $CaCO_3$ in a period of five moults, finding histopathology and all his experimental organisms died in the higher hardness. In his study, he did not differentiate between hardness and alkalinity and therefore the alkalinity could be high as well. Cripps and Nakamura (1979) showed no negative effect upon the survival of their prawns when they increased the hardness up to 500 mg L^{-1} as $CaCO_3$. In their study, they were using adult animals, and it is known that postlarvae are more susceptible to changes in calcium carbonate than adults and even juveniles (Sukadi, 1989; Latif, 1992).

While there was a good survival at alkalinity 250 and total hardness of 100 (88.9%), the organisms were showing low performance, they were almost without movement during the experimental time and producing the lowest growth rate in terms of percentage (25%). This indicates that high alkalinity reduces the growth, while low alkalinity (lower than 150 and preferably lower than 100) allows the growth of the postlarvae and their survival even if the total hardness is as high as 1000 mg L^{-1} as $CaCO_3$. Sandifer *et al.*, (1983) reported that high mortality of prawns in water of South Carolina have been related to total alkalinity levels above 180 mg L^{-1} as $CaCO_3$. They suggested that phytoplankton blooms at that level of alkalinity produce an increase in the pH resulting in the formation of a precipitate of calcium carbonate on the

exoskeleton of the prawns. They also mentioned that such a precipitate could obstruct the gills and therefore suffocate the prawns. Although the waters of the experiments of this thesis did not contain phytoplankton, the pH was unavoidably elevated when the alkalinity was high. Fujimura (1975) mentioned that calcium carbonate was precipitated on the exoskeleton of prawns reared at hardness of more than 300 mg L^{-1} as $CaCO_3$, and there was a high incidence of encrustation of *Epistylis spp.* (protozoans). Infestations by *Epistylis* spp. and *Vorticella* spp. have been reported to cause a whitish coloration in prawns (New, 1995). The suggested calcium precipitate was not observed in this series of experiments, but, in the section of incubation of embryos at high alkalinity levels (200 mg L^{-1} as $CaCO_3$) (chapter 4), the females developed a thin white film on the abdominal exoskeleton. Unfortunately, that white film was not analysed, but presumably it was a precipitation of calcium carbonate.

Prawn mortality can be due to ionic imbalance, high carbonates and low calcium for instance, instead of the high ionic concentrations. Stern *et al.,* (1987) mentioned that high magnesium concentrations could be the cause of the mortality of their experimental organisms. However, Funge-Smith (1991) mentioned that it could be the ionic imbalance instead of the high magnesium concentration, since in the experiment of Stern *et al.,* (1984) calcium concentrations were high as well.

Although the survival of the postlarvae at the lowest levels of both alkalinity and total hardness is not statistically different, it is quite low when compared with the next level of alkalinity 50 mg L^{-1} as $CaCO_3$. However, the

results of the growth rate did not demonstrate a negative effect of the low alkalinity on the postlarvae of *M. rosenbergii*. Survival of postlarvae in the experiments of Gonzalez Vera (1995) was low when alkalinity levels were higher than total hardness 25 and 50 over 20 mg L^{-1} (as $CaCO_3$) respectively. Similarly, Latif (1992) concluded that alkalinity higher than total hardness had a negative effect in postlarvae of *M. rosenbergii*. Vasquez *et al.*, (1989) suggested that extremely soft waters (20 mg L^{-1} as $CaCO_3$) are not optimum for *M. rosenbergii*. Furthermore, low concentrations of calcium carbonate ($CaCO_3$) can give free access for heavy metals to become toxic for aquatic organisms. That suggests that the presence of $CaCO_3$ is beneficial as a binding factor to minimise the effect of toxic metals, e.g. chromium and cadmium (Rosas and Ramirez, 1993). Therefore, a moderate $CaCO_3$ medium would be better for *M. rosenbergii* than a low one. Wurts and Perschbacher (1994) found that bicarbonate concentrations of 75 mg L^{-1}, in combination with a range of calcium concentrations from 20 to 250 reduced the copper toxicity as the calcium was increased. However, they mentioned that when the alkalinity was held at 20 mg L^{-1} as $CaCO_3$, calcium had no effect on the toxicity of copper causing a 100% mortality in catfish *Ictalurus punctatus*. Zimmermann *et al.*, (1995) found better survival, individual average weight and biomass for *M. rosenbergii* reared at alkalinity levels of 51 mg L^{-1} as $CaCO_3$, rather than either at 23 or 74 mg L^{-1} as $CaCO_3$ in combination with a 3% calcium content in the diets, rather than in 1.8%. They mentioned that there is a close relationship between the calcium of the water medium and the one in the diet, and therefore a necessity to control the calcium content in the diet according to the levels of calcium in the environment. However, Sukadi in 1989, found that there was no difference in

the survival of postlarvae of *M. rosenbergii* when these organisms were cultured with different calcium content in their diets (0.26 to 3.98%). Furthermore, Zimmermann *et al.*, (1995) did not find any interaction effect between the calcium content in the diet and the water hardness ranging from 18 to 300 mg L^{-1} as $CaCO_3$. *P. esculentus,* as many other crustaceans, stops eating and protects itself under the sand, at least for the first 6 hours after moulting and, after the exoskeleton is hard enough, then they start hunting for food. This suggests that calcium from the diet is not that essential for the species in the calcification process and instead, the environmental calcium plays an important role in the exoskeleton mineralization (Wassenberg and Hill, 1984). It is not clear why there was low survival of the organisms at the relatively low hardness and alkalinity levels of this study, since it has been reported that *M. rosenbergii* can live satisfactorily in acidic water with low calcium carbonate (Johnson, 1967).

The postlarvae that survived in the treatments of experiment III had a better growth rate than the previous two sets of experimental postlarvae (experiments I and II). However, all the postlarvae died in the treatments where the alkalinity levels were higher than 200 mg L^{-1} as $CaCO_3$. Those postlarvae that died after the first moult showed a white coloration, which appeared only between 12 and 36 hours prior to their death and usually in their late premoult or in the ecdysis stage. This is similar to that described by Sandifer *et al.*, (1975); Delves-Broughton and Poupard (1976) and Latif (1992). The organisms in this study, however, did not show the symptom immediately after being transferred to the different alkalinity and total hardness concentrations, but after

few days of being reared in them. It is unclear why this happened in that way and not suddenly as is reported in other works. Numanoi (1937) mentioned that *L. exotica* showed a whitening coloration during the shedding of the old exoskeleton, and he mentioned that that coloration was due to calcium in the proteins of the haemolymph. Harrison and Martin (1954) found a similar pattern in the moulting cycle of *Limnoria lignorum*, where they interpreted the changes in the whitening of the organisms as changes in the calcium concentration in their organs throughout the moulting cycle. Latif (1992) described that white coloration in some postlarvae and juvenile of *M. rosenbergii,* and he suggests that high alkalinity (> 100 mg L^{-1} as $CaCO_3$) was the causal agent for it. Sandifer *et al.*, (1975) found the whitening coloration in the postlarvae of *M. rosenbergii* just before the animals were to die in high salinity concentrations (> 30 ‰), and Delves-Broughton and Poupard (1976) mentioned that white syndrome has been observed in *M. rosenbergii* as a characteristic of poor control of the husbandry.

Density could not be the factor for the white coloration, since the density was much less than that recommended for new postlarvae rearing (5 to 100 organisms L^{-1}) (Sandifer and Smith, 1985), being only 3 postlarvae per 8 L in the experiments of this study. Several authors cited in Nash *et al.*, (1987) mentioned that this symptom is, *per se,* a response to environmental stressors like sudden fluctuation in temperature, salinity, overcrowding, exposure to direct sunlight or air, and direct handling. Furthermore, Nash *et al.*, (1987) found the same white coloration and they mentioned that it is possible that lack of dissolved oxygen due to overcrowding was the causative factor for that. The

levels of oxygen in the present study were high above that recommended for the species, so it is unlikely that the symptom was caused by oxygen insufficiency in the system, neither by sudden changes in the temperature and salinity, direct air or sun light exposure, since those factors were well under control in this study. It seems to be a clear indication that the whitening of the postlarvae is a result of stress caused in the organisms, however, it is not so clear what this may be.

A possible reason for that stress in the organisms could be intoxication by calcium. Greenaway (1985) mentioned that the storage of calcium is mostly in the form of phosphates and not in carbonates in the hepatopancreas of some marine crustacean, i.e. *C. maenas*, *C. sapidus* and *P. argus.* He mentioned that this is due to the lack of phosphorus in the marine environment and the abundance of carbonates. Indicating that carbonates could be substituted immediately and phosphorus not, so then the animal stores the necessary ions. However, it is calcium carbonate that is stored in high quantities in the haemolymph of freshwater and terrestrial crustaceans when they are in low environmental carbonate media. If this accumulation of calcium is above the requirement for the organisms and possibly exceeding the carrying capacity of the blood then, perhaps, that induces the whitening appearance on the prawns just before they died. Suggesting that, if the animals were in high carbonate waters, they could have had problems to get rid of the excessive internal calcium carbonate that the organisms were acquiring from the media through a period of time. That is explained by the process of taking water and ions from the medium through membrane transport. Paul and Sharpe (1916) mentioned

that, in *Cancer pagurus* (taken directly from the sea for sampling), the amount of haemolymph was highly increased immediately after moulting, but they said that the amount of calcium was practically constant. According to Rubin (1982) calcium is a very important element, since it helps in the process of initiating and maintaining life during the fertilisation of an egg and all the way through cell growth and development. He also mentions that calcium helps to control cell injuries and death. Therefore, when the cell membrane is damaged, an influx of calcium can disturb the intracellular calcium homeostasis, giving as a result an accumulation of calcium at high levels that could be toxic and even cause the death of the cell. It may be possible then, that in some of the fast moulting cycles, the postlarvae could have been damaging and even tearing apart the membranes of some of the membranous layer cells, inducing an uncontrolled calcium influx when the organisms were taking liquid into their systems to increase their volume, resulting in the white coloration, intoxication and followed by a quick death.

It is interesting to mention that in the treatment with 250 mg L^{-1} as $CaCO_3$ of alkalinity and 1000 mg L^{-1} as $CaCO_3$ of total hardness, the postlarvae managed to survive until the third moult after the experiment started and before the appearance of the white coloration. The moult frequency up to then was similar to the moult frequency of the postlarvae held at 100 mg L^{-1} as $CaCO_3$ for both alkalinity and total hardness (6.2 ± 0.3 days and 6.4 ± 0.6 days respectively). That suggests that the moult frequency could be dependent on the metabolic fitness of the postlarvae. However, postlarvae may take some time to show effects of stressful experimental conditions even up to the third

experimental moult (Brown, J.H., personal communications, 1999). Thus suggesting that the survival of those stressed postlarvae up to the third experimental moult was perhaps a merely time of reaction to the effects of both the high total hardness and alkalinity of the water media. In the results of Sukadi (1989) there was no significant effect of the high hardness in the survival of the postlarvae of *M. rosenbergii* until week 6th of his experimental time (18 weeks). He mentions that a physiological change occurs after the postlarva reaches the 9th week of development or that accumulation of calcium reaches slowly critical levels around that week.

All the postlarvae died in the treatments where the alkalinity was higher than 250 (experiment III), and most of them died after the acclimatisation moult and only a few (4 organisms) survived the first experimental moult. Schmitt and Uglow (1993) reported that sudden changes in temperature are more stressful to *M. rosenbergii* than changes over longer periods of time, they mentioned that it could be that the prawns acclimatise themselves to these gradual temperatures changes (18 to 26 °C.). They measured the total ammonia and total nitrogen efflux rates as indicators of the stress caused by changes in the temperature. That could be an explanation for the sudden mortality of the postlarvae that died at the beginning of the experiments, since they were taken out of the water to be dried and weighed prior to the experiments. During that process, the organisms were kept out of the water for about 30 seconds and then transferred to a different water system varying in chemical composition (section 3.2.3. and 3.2.4.). Changes in the temperature were not as high as in the report of Schmitt and Uglow (1993), but, a difference

of 2 ± 1 °C. occurred, plus the changes in the chemical concentration were high and acute. Although the changes in the temperature seem not to be the source of mortality, the changes in the chemical composition of the water media could cause a similar reaction as to the one described by high temperature changes. However, not all the organisms died immediately, suggesting that only the physiologically weak did not tolerate the changes and perished.

As mentioned before, changes in total ammonia are measured as indicators of stress caused by changes in temperature, but, ammonia itself can increase mortality by the toxicity of unionized ammonia. New and Singholka (1982) said that high pH levels in freshwater prawn ponds are responsible for mortality, both because of the direct effect of the pH and the greater solubility of waste ammonia at high pH. Kumari and Pandian (1991) found that ammonia excretion increased in the postmoult stage of *M. nobilii* and they suggest that it was due to the breakdown of proteins. This could increase the stress present in each organism at the time of moulting and therefore making the organism unable to cope with the stress provided by ion regulation. Total ammonia levels up to 1.6 mg L^{-1} are considered to be safe for *M. rosenbergii*, giving a 0.1 mg L^{-1} of unionized ammonia at 12 ‰, 28 °C. and a pH of 8 (Wickins, 1976a). Wickins (1976b) reported that a maximum of 0.7 mg L^{-1} of total ammonia in recirculation systems used in their experiments was not harmful for the growth of *M. rosenbergii*, and that is much higher than the maximum level obtained in this experiment (< 0.3 mg L^{-1}). Consequently, it could be suggested that total ammonia was not the cause for high mortality or low growth rate. *M. rosenbergii* tolerates in equality to *Penaeus* spp. the concentrations of ammonia, but nitrites

are more toxic to the former species (Wickins, 1976b). However, he mentioned that nitrites increment their toxicity when there is a lack or reduction of inorganic CO_2 in recirculation systems, due to the lack of inorganic carbon necessary for the reactions in which *Nitrobacter* spp. and *Nitrosomonas* spp. reduce the toxic nitrogen. However, that is not the case in this study, since a flow through water system was used and elevated amounts of inorganic carbon were in the system due to the high CO_3^{2-} concentrations. The maximum level of nitrite in this study was 0.008 µg L^{-1} and that was not believed to be the reason for the mortality, since Wickins (1976b) reported a safe level of 1 mg L^{-1} for the species.

Sarver *et al.*, (1982) found that postlarval mortality was high when *M. rosenbergii* postlarvae were transferred from the hatchery to the grow-out rearing pond. However, they found high variability in the mortality from pond to pond and at different stocking densities. They mentioned that the postlarval fitness is affected by genetic background, larval history and water quality in the ponds. That suggests that the mortality of the postlarvae in this study was directly affected by a particular factor, and that is the ionic differences in the different treatments, total hardness and alkalinity.

3.4.4. Moult frequency

From the results of the moult frequency in experiment I, it is clear that a shorter moult cycle was produced in postlarvae held in higher total hardness levels. In experiments II and III no differences were found. However, after the

transformation of the data into percentages, all the postlarvae showed a general trend to increase their moult frequency as the total hardness and alkalinity increased. A similar response was found by Brown *et al.*, (1991) and Latif (1992). However there was some ambiguity in the results of Brown *et al.*, (1991) in that size ranges of animals was quite large so differences in moult rate could have been connected with size differences as much as with hardness and alkalinity effects. Also Cripps and Nakamura (1979) reported an opposite finding, i.e. a reduction in the growth rate and moult frequency or lengthening of the moult cycle when their organisms were reared in high calcium concentrations (up to 500 mg L^{-1} as $CaCO_3$). Kleinholz (1941) mentioned that the crab *Uca pugilator* regulates the amount of calcium in its exoskeleton by the duration of the intermoult period. He also mentioned that if the moult is delayed due to inanition, the level of calcium in the exoskeleton rises up to an 86%. Therefore, the increase of the moult frequency observed in this study could be targeting the excretion of the excessive calcium carbonate accumulated in the organisms, especially in the exoskeleton when the postlarvae were held at high environmental calcium carbonate concentrations.

The postlarvae that died after the third moult in experiment III had a similar moult frequency (6.1 ± 0.2 days) to the other postlarval groups, which survived in the same experiment. It is not clear why the organisms survived up to that moult and they did not die immediately on or after the first moult. Presumably, in experiment III, the moulting frequency increased until the animals could not cope with the stress and died at the extreme conditions of alkalinity (250 mg L^{-1} as $CaCO_3$) and total hardness greater than 100 (mg L^{-1} as

$CaCO_3$), due to a possible cross over effect caused by the combination of both high total hardness and high alkalinity. Sukadi (1989) suggested that there could be a physiological change around the 9th week after metamorphosis in the postlarvae. The postlarvae used in these experiments were collected after six weeks from metamorphosis and they were moulting almost once per week, so that makes a total of 9 weeks after metamorphosis, time suggested by Sukadi (1989) for the physiological change. Therefore, it could be that those organisms in the adverse conditions where unable to cope with the stress caused by the ionic imbalance during such a suggested physiological change.

It was shown in Figure 10., that some postlarvae groups of the three different experiments were having a higher moult frequency since the first experimental moult giving a slight difference in the average moulting frequency for the whole experimental period. That suggests that either the postlarvae were moulting at a different frequency even before the experimental stress caused by the alkalinity and total hardness was applied or that they reacted to that stress within the first moult. It is not clear whether the organisms that died after the third experimental moult were affected by their own physiological changes at that stage of development, or as mentioned above, they could have been moulting at different frequency since before they experienced the changes in total hardness and alkalinity. Therefore, more research is needed to identify the period and cause of that physiological change in the postlarvae at the 8-9th post-metamorphosis week.

Prawns in the higher alkalinity levels moulted more often and their growth rate was smaller when compared with the postlarvae hold at lower level of alkalinity. This was similar to that reported by El-Gamal (1987) and Brown *et al.,* (1991) who found that prawns could moult and lose weight instead of gaining it when reared in high total hardness. Similarly, postlarvae of *M. rosenbergii* could moult, but they were losing weight when they were reared in waters containing different aluminium concentrations (Baker, 1989). Cameron and Wood (1985) demonstrated that reducing the pH in the sea water, reduced the HCO_3^- concentration, as well as the apparent flux of H^+, but when Pco_2 was increased then, the HCO_3^- also increased together with the H^+ flux, but the pH decreased. These authors indicated that mineralization of *C. sapidus* was more dependent on the variations of the HCO_3^- than in those of the H^+. They also found that the pH of the liquids of the exoskeleton is 0.3 to 0.5 higher than the one in the haemolymph of *C. sapidus.* Indicating that the organism is constantly transporting alkaline elements ($CaCO_3$) to the exoskeleton, and that process could be seen as a strategy to induce moulting once the pH of the liquid in the exoskeleton is similar to the one of the haemolymph. However, Cameron (1989) mentioned that the timing in the control of the physiological and behavioural events related to H^+, HCO_3^- and Ca^{2+} fluxes could be influenced by a series of hormonal signals. Therefore, if pH is too high in the external media, then the organism will have problems to excrete its excessive internal accumulation of calcium carbonate. This being caused by an electrochemical balance pursued by the organism to excrete H^+ ions in order to compensate the $CaCO_3$ intake (Cameron, 1985b), unless the organism could manage to increase its moulting frequency and shed the old exoskeleton constantly. However, increasing the

moulting frequency will imply large energy consumption and possible losses of other valuable materials like proteins and chitin, which consequently will reduce the potential of the organism to grow. On the contrary, if the external media has a low pH value then, the exoskeleton would decrease its calcium carbonate content and therefore its rigidity letting the organisms not only exposed to metabolic failures, but under potential predatory action. Wright (1979) found that *Gammarus pulex* had 94% of its body calcium in the exoskeleton at the intermoult stage, and Wright (1980) demonstrated that that species lose 54% of its corporal calcium with the exuviae and then proceeded to have a rapid calcium uptake from the external media after the moult. The haemolymph calcium content remains lower than intermoult stages after moulting, whilst the hepatopancreas is over saturated with calcium reserves at the immediate postmoult stage decreasing to intermoult levels at postmolt stage. Therefore, if the prawns were having an excess of calcium and magnesium in their carapace then they would probably try to reduce it by increasing their moult frequency.

Howe (1981) found that 96% of the moults occurred during the dark period of his experiment. He also found that moulting frequency was increased as the time of the animal were without renewed freshwater in the tanks, and as soon as the water was changed or renewed the moulting frequency was reduced. That suggests that the stress caused by some factors like ammonia, or poor dissolved oxygen is a sufficient cause to induce the moult in the freshwater prawn. However, his results were from prawns with a high variability in size (0.17 to 2.84 g). He described a triggering effect in the moulting synchrony, i.e. when one organism moulted the others tried to moult as well,

perhaps to avoid cannibalism. In the present study, the organisms were separated in replicates of three in different trays, not allowing contact of the same water, since it was a flow through water system. Therefore, the triggering effect is less likely in this study. Howe (1981) suggests that the moulting activity represents the responses of the postlarvae to an intermittent external stimulus, and in the present study, the only external stimulus was the ionic variation (total hardness and alkalinity) in the water media.

3.4.5. Carapace mineralization

In a study with crabs (*Podophthalmus vigil*) Sather (1967) found that the carapace was the part of the exoskeleton that showed greatest variation of inorganic calcium content within the moult cycle. Thus one of the main reasons for studying this part of the exoskeleton during the mineralization process of the moult cycle, plus its easy collection and measurements on the small postlarvae of *M. rosenbergii*.

Osmoregulation of a marine crustacean kept in a non isosmotic medium can cause a waste of energy by excessive ionic regulation (Funge-Smith, 1991), which could be the same case for larvae of *M. rosenbergii* growing in brackish water and even early metamorphosed postlarvae. If that occurs then, the performance of the organism could be affected in many ways, i.e. growth rate, survival, moult frequency or even the balance and content of different internal ions. Calcium and magnesium concentrations of the cast and

intermoult carapaces were studied in this work as complementary tools for that performance of the postlarvae.

Similarly to the growth rate, there was no significant difference among the length-specific, dry weight of cast or intermoult carapaces within the different treatments of experiment I and II. However, in experiment III, when alkalinity increased at 250 mg L^{-1} as $CaCO_3$ or above, the response was a significant decrease of the length-specific dry weight (in both cast and intermoult carapaces) as well as the growth rate, and an increase of the moult frequency. When the levels of total hardness were as high as 1000 mg L^{-1} as $CaCO_3$ there were no differences, indicating that alkalinity was the influencing factor in the reduction of both, growth rate and length-specific dry weight. In a similar study, Latif (1992) found that the length-specific dry weight of postlarvae was lower as the alkalinity increased, and Brown *et al.,* (1991) found that an increase in the size and length-specific dry weight of postlarvae was positively related. However, in the latter study they found that total hardness ranging from 31 to 71 mg L^{-1} as $CaCO_3$ did not influence the length-specific dry weight of the postlarvae. Similarly, in this study, neither a significant difference nor a trend was detected at low levels of alkalinity (100 or less mg L^{-1} as $CaCO_3$). However, intermoult carapaces proved to be heavier than cast ones in all the three experiments. In experiment III, cast carapaces of postlarvae held at moderate alkalinity (100 mg L^{-1} as $CaCO_3$) had only 30.4% of the intermoult length-specific dry weight, whilst at alkalinity levels of 250 the cast carapaces had 52.4% of the intermoult ones. Those differences in the length-specific dry weight of cast and intermoult carapaces were similar, 30.4 and 27.7% when

total hardness increased from 100 to 1000 mg L^{-1} as $CaCO_3$ respectively. It seems then that postlarvae were more efficient at reabsorbing materials from the old exoskeleton in moderate alkalinity levels than from high ones regardless of the total hardness.

The results of the calcium content did not indicate a clear trend in low to moderate alkalinity levels. However, in experiment III, the response was similar to the length-specific dry weight, where calcium of the carapace decreased as alkalinity increased from moderate (100) to high levels (250) in both cast and intermoult carapaces, though there was not much difference and statistically they were not significantly different. When calcium was related to the length of the carapace (length-specific calcium content), the calcium content was significantly lower at high alkalinity levels than at moderate ones, for both cast and intermoult carapaces. That suggests that the postlarvae were able to moderate their calcium content even at moderate levels of alkalinity (100 mg L^{-1} as $CaCO_3$). The length-specific calcium content showed that intermoult carapaces had more calcium than the cast ones. This could indicate a withdrawal of the organic matter from the exoskeleton previous to ecdysis. El-Gamal (1987) found that during premoult, the calcium content of the carapaces of *M. rosenbergii* was significantly higher than in the intermoult stage. However, he suggested that this increment of calcium in postmoult exoskeletons, was due to the removal of both organic and inorganic materials from the procuticle and, therefore, that reduced the carapace mass, giving a net increment of the calcium content, which is similar to the described for the postlarvae of this study held at high alkalinity levels.

Haefner (1964) indicated that the calcium ratio content between haemolymph of *C. sapidus* and sea water was different from intermoult to premoult and postmoult stages, being always high in premoult, moderate in intermoult and low in postmoult haemolymph. Morgan and McMahon (1982) found that environmental acidic conditions produced an increase of the haemolymph calcium content in *P. clarkii* and *O. rusticus*. They suggest that the increase of internal calcium content was due not to high environmental calcium concentrations but to a dissolution of exoskeletal calcium carbonate in order to compensate the acidosis. Wickins (1984) found that *P. monodon* reduced its content of calcium in the cast carapaces, but not in the intermoult ones, when it was reared at lower pH water. However, Fieber and Lutz (1982) indicated that the total calcium of the body decreases during premoult. Therefore, it could be that the lower length-specific calcium content of the cast carapaces was due to a loss of calcium to the environment during the ecdysis. Simkiss (1976) said that the mitochondria is the bridge by which calcium is transported to the areas where is needed for mineralization in crustaceans, and carbonates although able to be transported, are more difficult and require much more energy to accomplish it. If that is the case in *M. rosenbergii*, then it could be that metabolic CO_2 would be used more easily by the mitochondria for the mineralization process, rather than CO_3^{2-}. This could suggest that, if postlarvae were reared in a high calcium carbonate media, then they would be unable to cope with the regulation of CO_3^{2-}, rather than with an excretion of calcium via ecdysis, making alkalinity the most affecting factor of their development rather than the total hardness.

Magnesium content in the cast and intermoult carapaces was always much lower than calcium in all the treatments. A higher concentration of magnesium was detected in carapaces of postlarvae held at high environmental magnesium concentration (total hardness of 1000 mg L^{-1} as $CaCO_3$). However, when alkalinity increased from 100 to 250 (mg L^{-1} as $CaCO_3$), there was a significant increase of magnesium content in both cast and intermoult carapaces. Hagerman (1980) mentioned that internal magnesium concentrations of *Crangon vulgaris*, were always maintained below the external environmental magnesium. However, he mentioned that urine magnesium concentrations were always higher than the external concentrations. Similarly, Stern *et al.*, (1987) found that *M. rosenbergii* maintained blood magnesium hypoionic to the media. That could explain why the magnesium concentrations were always lower than calcium in the intermoult and cast carapaces of *M. rosenbergii*. The lowest magnesium concentration could be due to the equilibrium that has to exist within the haemolymph, therefore, if the haemolymph is retaining calcium to supply the requirements of the exoskeleton when hardening, then the less necessary magnesium ions should be excreted, to reduce the excess of cations.

Magnesium could interfere with enzymatic activity in the muscle and hepatopancreas (Romero, 1990), so this could be another reason why *M. rosenbergii* always keeps this cation at low concentrations. Wickins (1984) found that the weight of the carapace of *P. monodon* was less at low environmental pH than at a higher one, and the same response was observed for magnesium concentration in the carapaces. In this study the results of the

length-specific magnesium content did not show any trend on magnesium concentrations when the alkalinity was changed. However, the length-specific magnesium content responded in a similar way to the magnesium content in the carapaces, showing an increase as the environmental magnesium increased within the total hardness, and the intermoult carapaces always had more length-specific magnesium content than the cast ones. That agrees with the early suggestion that postlarvae were reabsorbing organic material from the exoskeleton prior to ecdysis.

It is possible to find differences of the calcium and magnesium content in *M. rosenbergii* reared at different water hardness and alkalinity, but care should be taken to identify the moulting stages of the organisms, since changes in the ionic content of an individual organism occur during the moult cycle. Therefore, a carapace sampled from an organism reared in low calcium carbonate water for example, which was in its intermoult stage, most probably will have a higher calcium content than one of an organism reared in high calcium carbonate, but which was sampled in the premoult stage of its moulting cycle.

Calcium and magnesium levels were expected to be higher in organisms kept at higher environmental ionic concentrations. However, apart from the third experiment where the alkalinity levels were extremely high (250 mg L^{-1} as $CaCO_3$ and above) there were no differences at low or moderate alkalinity and total hardness levels. Therefore, some aspects related to the technique used in this study are discussed, since they could have had an effect

in the measurements of the ions in the carapaces of postlarvae at low or moderate alkalinity and total hardness levels.

Although some organisms were shedding their exoskeletons due to the effect of the ion imbalance, it was expected that they would harden their new exoskeleton as fast as possible in order to excrete the excessive calcium carbonate from their internal system when they were held in higher ionic water concentrations. However, some collected carapaces, from both cast and intermoult ones, were very soft and thin. In respect to that, France (1987) found that *O. virilis* reared in acidified waters (pH 5.4 to 5.6) reduced the rigidity of their exoskeleton and the calcium content was 25 to 35% less than the control organisms, whilst tissue concentrations of manganese and mercury increased. That presumably could have been done due to loss of calcium carbonate from the exoskeleton to the medium. However, in the experiments of this thesis, the pH was never low enough to cause such an effect on the exoskeleton of the postlarvae. Paul and Sharpe (1916) mentioned that if a premoult crab is prematurely removed from its old exoskeleton, it produces a leathery new one, but this will not calcify. That could be related to the fact that most of the calcium carbonate in the old exoskeleton was removed when the old exoskeleton itself was shed, prohibiting the crabs to remineralize their new exoskeleton. It could be that some of the experimental postlarvae used in this study, prematurely moulted by the stress, caused by the water media, were not able to regulate and balance their ionic content, resulting in a similar inefficiency to completely remineralize their new exoskeleton.

Hilton *et al.*, (1984) mentioned that different ion extraction techniques of exoskeletons, could give relatively large differences for the analysis of inorganic ions. The high variability in the results of the ionic content of the exoskeletons analysed in this thesis could have been caused by the content of organic material in cast and intermoult carapaces. It was noticed that some of the carapaces were thinner and softer than others plus the high variability in the size of the individual postlarvae gave a high variation in the size of the carapaces themselves. As mentioned earlier in the methodology, the intermoult carapaces were carefully dissected from the animal using dissection equipment, removing as much as possible of the membrane or muscle attached to the inner side of the carapace. However, in a slide of an histological cut (see Appendix III,b for details of the histological method for calcium) it is easy to see how difficult it could be to completely remove the membranous layer from the intermoult carapace (Plate 12.), due to its closeness to the mineralized part of the carapace, whilst in late premoult carapaces

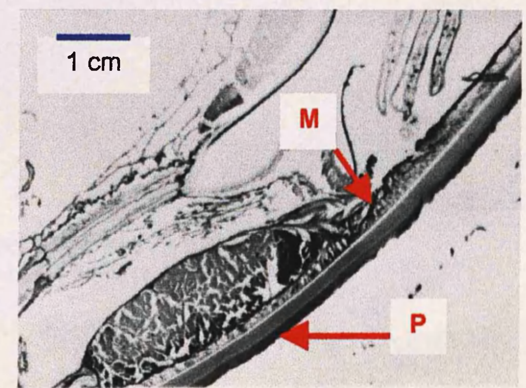


Plate 12. Intermoult carapace.
M = membranous layer; P = Precdysal cuticle; (1 cm = 35.3 μm).

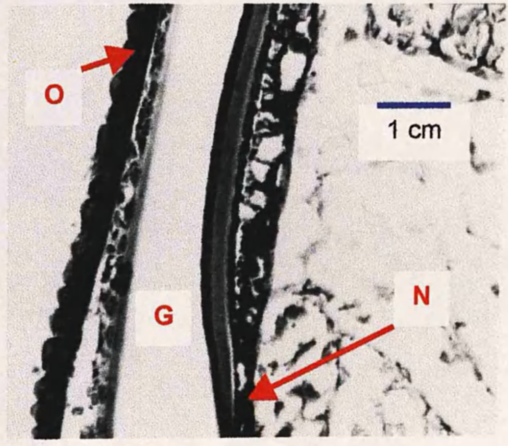


Plate 13. Cast carapace.
O = old carapace; G = inter-carapaces space; N = New carapace; (1 cm = 17.7 μm).

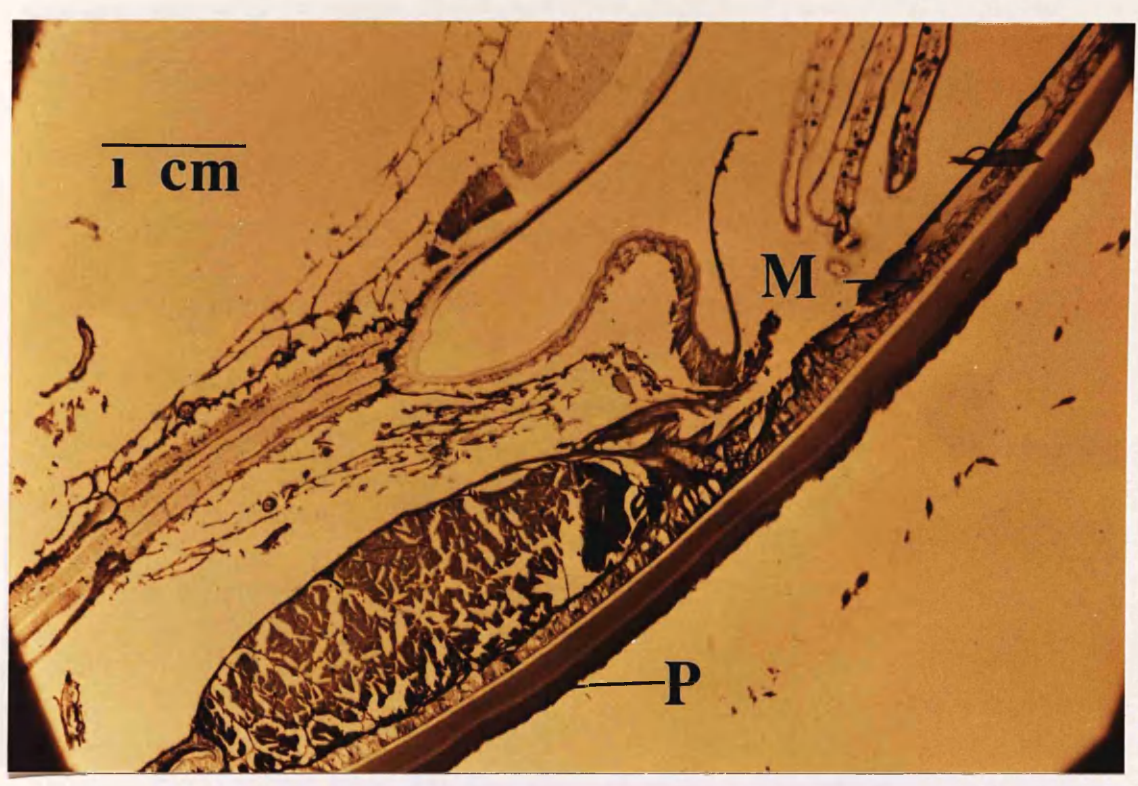


Plate 12. Intermoult carapace.
M = membranous layer; P = Precdysal cuticle; (1 cm = 35.3 μm).

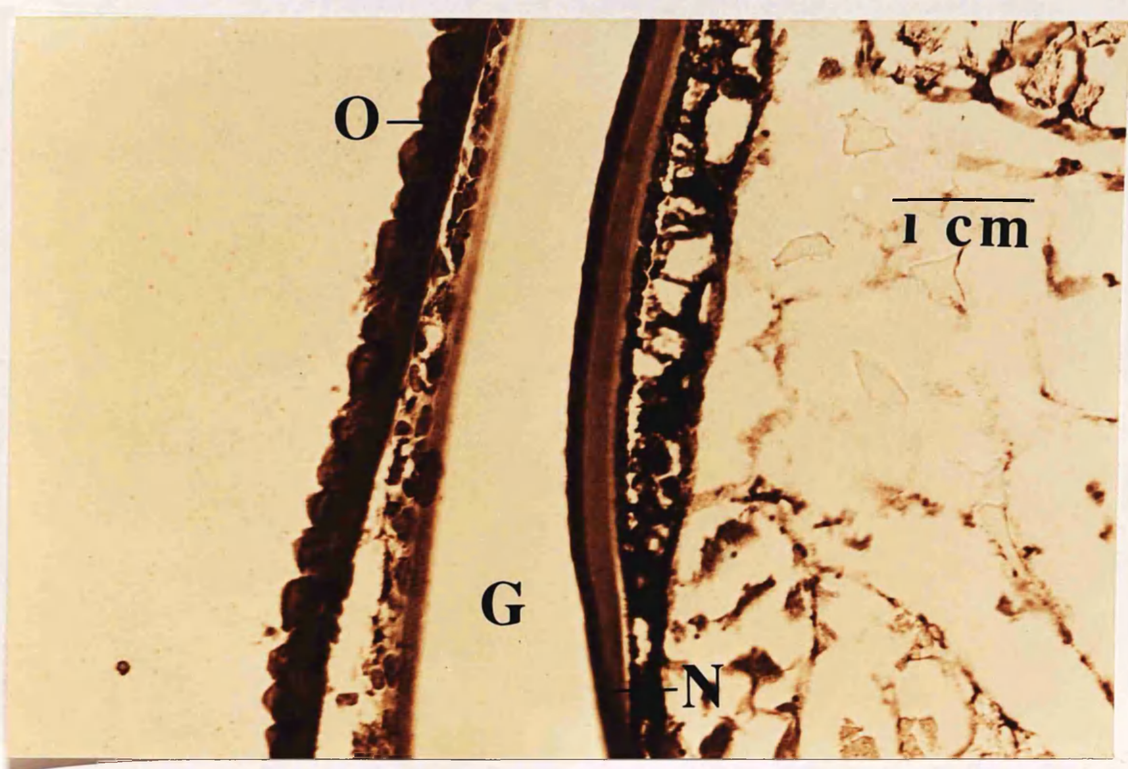


Plate 13. Cast carapace.
O = old carapace; G = inter-carapaces space; N = New carapace; (1 cm = 17.7 μm).

that membrane is removed naturally (Plate 13.). Therefore, it was possible that accidental small pieces of membranous tissue invisible to the naked eye were left attached to the carapace serving as traps for calcium and magnesium ions, which could be in transit from the carapace to the storage sites and *vice versa*, depending on the exact time of the dissection.

The intermoult period was determined as half of the period in time between the moult previous to the last and the last one. In order to be sure that this was the case, some organisms were sampled prior to the actual experiments, and indeed the mid time between the two moults proved to be the intermoult period for postlarvae held in the Tropical Aquarium water system. However, the groups of postlarvae held in the experimental waters were under different alkalinity and total hardness and that definitely altered their metabolism as has been demonstrated by the differences in the survival and growth rate. The moult frequency was altered as well and, as shown in Figure 10., it was getting more and more different as the postlarvae were held either at low total hardness and alkalinity or at high levels. However, that difference of the moult frequency was growing gradually after each moult. Therefore, if for example, the intermoult period previous to the one selected for the sacrifice of the postlarva was 4 days after the moult then, the next one could probably be 3.7 or 3.5 days and not at 4 as the previous one in the treatments with high ionic concentration in the water and *vice versa* for low concentrations. Thus suggesting that a variation in the concentrations of both calcium and magnesium in the carapaces could be produced as a result of those small variations in the time of dissection of the carapace. Furthermore, if some postlarvae were bigger than others, that

could increase the variability in the ion concentration per set of postlarvae in each treatment. Unavoidably that was the case and the reasons for the individual differences in growth have not been fully understood yet. There were no significant differences in the ion concentrations between the postlarvae of the different treatments of each experiment due to the variability in most of the results of the carapace mineralization. However, as discussed before, there were big differences among the cast and intermoult carapaces in all the three experiments.

It is important to mention that some of the differences in calcium and magnesium concentrations could have been due to the differences in the thickness of some of the carapaces from some postlarvae in the same trial, since the carapaces that were thinner were soft as well, suggesting a lack of calcium carbonates and in less extent magnesium carbonates, which give rigidity to the carapace structure.

3.5. CONCLUSIONS

Growth rate of postlarvae of *M. rosenbergii* is reduced as the alkalinity increases, and this effect is more acute at levels higher than 200 mg L^{-1} as $CaCO_3$. Total hardness from 20 and up to 1000 mg L^{-1} as $CaCO_3$ do not affect the growth rate of the postlarvae if the alkalinity is lower than 100 mg L^{-1} as $CaCO_3$.

Survival of the postlarvae of *M. rosenbergii* is severely affected by increases in the alkalinity of the water media. Alkalinity above 250 is lethal for the postlarvae of the species. Total hardness has no effect up to levels of 1000 mg L^{-1} as $CaCO_3$ in the survival of the postlarvae when the alkalinity is lower than 200 mg L^{-1} as $CaCO_3$. The increase of total hardness at levels of 1000 reduces the survival of the postlarvae when the alkalinity is equal or above to 200 mg L^{-1} as $CaCO_3$.

The moult cycle of postlarvae of *M. rosenbergii* gets shorter as both total hardness and alkalinity increase.

High alkalinity level reduces the length-specific dry weight of cast and intermoult carapaces as well as the calcium content. Contrastingly, magnesium content of the carapaces increases as the alkalinity and total hardness increases.

CHAPTER 4

4. EFFECTS OF *M. rosenbergii* EMBRYONIC ACCLIMATISATION TO DIFFERENT ALKALINITY ON ITS LARVAL AND POSTLARVAL DEVELOPMENT

4.1. INTRODUCTION

High hardness and alkalinity had a great detrimental effect in the development of the postlarvae of *M. rosenbergii,* but it was shown in the previous chapter that although hardness had a negative effect in the development of the postlarvae, alkalinity had a greater influence on them. Although there is a range of chemical parameters in different freshwater reservoirs with potential for the culture of the species, it has been demonstrated that some of them are not appropriate for the culture of the species, when the alkalinity levels are above the 250 mg L^{-1} (as $CaCO_3$). These conditions rule out many areas for the development of the prawn industry, for example some parts of the southeast part of Mexico, which have an alkalinity level as high as 840 mg L^{-1} as $CaCO_3$ (Flores Nava, 1990). However, it was worthwhile to study a possible acclimatisation of the organisms to a higher environmental alkalinity level due to the low levels of alkalinity affecting the development of the postlarvae (from 150 to above 250 mg L^{-1} as $CaCO_3$). That approach was preferred rather than to try to reduce the levels of alkalinity itself, since it would be impractical and almost impossible to do on a large scale, due to the high cost implied and the chemicals used for it. Genetically-modified organisms to tolerate higher levels of that parameter, would be a long term research and

experimentation that besides might imply many unknown side effects that could affect negatively the environment and the species itself.

M. rosenbergii is very susceptible to temperature changes, so studies of the temperature range alterations are very important, since possible acclimatisation to a wider range of temperature could increase the range of culture of the species. Gomez Diaz (1987) worked in the acclimatisation to different temperature of the embryos of *M. rosenbergii*, finding a positive response where the embryos could develop and survive in a wider range of temperatures, 25 and 31 °C., instead of 29 °C. when they were not acclimatised, increasing the potential to produce the species in a wider temperature range. However, acclimatisation studies have not been studied very fully. Apart from salinity in postlarval and adult organisms, there have been very few works in acclimatisation of the species to different environmental parameters, and almost none in the embryonic stages of it. However, acclimation to different environmental factors in different aquatic species has been conducted to try to improve their growth rate and survival in rearing conditions, which are different to their natural environment. For instance Jeberg and Jensen (1994) acclimatised the crayfish *A. astacus* to different temperatures to identify its effect on the nitrite accumulation in the haemolymph, finding that the rise of the temperature increased the accumulation of nitrite. Thus causing an imbalance in the ionic homeostasis of Cl^- and K^+ in the muscle of the organisms and therefore a perturbation in the metabolic process. They also found that K^+ rose in the midgut gland tissue, whilst it was reduced in the muscle tissue, indicating an over-activation of the excretory mechanisms of the

midgut gland. Farmers in the Netherlands have seen that eels acclimatised at low pH (5.8) grow better than the ones that are reared in ponds with artificially elevated pH (Van Der Meer, M., personal communications, 1997). Samuel *et al.*, (1997) studied the acclimatisation of *M. malcolmsonii* to different salinity concentrations, and he found that a reduction of the embryonic incubation period was achieved in lower salinity.

One of the external conditions that could have an effect in the embryonic development is the alkalinity concentration found in the water in which the incubation period is carried out. If the alteration of the optimal levels of alkalinity is so high that the embryo reduces its development in order to survive and finally to reach the larval stage, it would probably die in a short period of time or just grow at a very slow rate. However, the embryo could acclimatise to higher or lower levels of alkalinity in the water, which could transfer its upper or lower limits to higher or lower concentrations of alkalinity at the time of the metamorphosis of the larvae into postlarvae. Kinne (1964) describes the adaptation as "*the adjustment at the functional level that brings about an increased efficiency of performance*". Therefore, if for example the optimal range of alkalinity for a good development of the embryo is between 50 and 100 mg L^{-1} as $CaCO_3$ then, perhaps, the adaptation of the embryos to a higher concentration of alkalinity could go up to 150 mg L^{-1} as $CaCO_3$. As mentioned earlier, Gomez Diaz (1987) demonstrated that the embryos of *M. rosenbergii* were successfully acclimatised to a sub-optimal temperature of 25 °C., producing healthy larvae. In that case it could be assumed that the sub-optimal temperature (25 °C.) was not low enough to produce stress but instead

produced an adaptation. The results of chapter 3 showed that high environmental alkalinity levels had a negative effect in the growth rate and survival of postlarvae of *M. rosenbergii.* Therefore, an acclimatisation experiment was conducted to analyse a possible acclimatisation of the embryos to higher concentrations of carbonate in the water and to see if their resulting postlarvae develop a better performance in higher environmental alkalinity. Total hardness was not altered, since Sukadi (1989) suggested that the optimal level of hardness for the embryos of *M. rosenbergii* to hatch satisfactorily is around 150 mg L^{-1} as $CaCO_3$, rather than low or high levels (18 and 300 mg L^{-1} as $CaCO_3$ respectively). Furthermore, in this study it was seen that alkalinity by itself was a more stressful agent than total hardness, unless both parameters were at elevated concentrations causing a croos over effect.

4.1.1. Embryonic development

As mentioned earlier in section 1.4.1.1., the embryos of *M. rosenbergii* can hatch as larvae after 17 days of incubation, or longer depending on the environmental conditions as well as their genetic history (Ling and Merican, 1961; Ling, 1969; John, 1957). If the external conditions and the health of the progenitor are good then, the embryo would develop in a normal healthy way (Gomez Diaz, 1987; Wickins *et al.*, 1995), unless they have a genetic problem. If the health of the progenitors is not good enough, then there will be a lack of healthy nutrients and components in the embryo itself, giving as a result a weak or abnormal embryo (Wickins and Beard, 1974). If the embryo alters its

metabolic rate in order to compensate for the changes in the external conditions (i.e. temperature, salinity, dissolved oxygen, photo-period, hardness, etc.) that could have an effect on its own development (i.e. reducing its growth rate and ability to use energy from the yolk), leading perhaps to the formation of a poor quality larva or the possibility that it will not even hatch into one. Wickins *et al.*, (1995) found a similar response to that, but in embryos of the lobster *H. gammarus* exposed at low salinity.

4.1.2. Energy consumption as a measurement of stress

According to Balasundaram and Pandian (1982) the energy content of a freshly-laid embryo of *M. nobili* is 0.19 calories, a newly hatched larva 0.14 and a starving, 8 days larva contained only 0.09 calories. They mentioned that 73.7% of the total embryonic energy is utilised for the body substance of the zoea, by the time this is ready to hatch. However, an accelerated consumption of energy could theoretically occur when the embryos are incubated in adverse environmental conditions. Thus having a negative effect on the development of the embryos themselves and perhaps not allowing themselves to reach the larval stage. Wickins *et al.*, (1995) found that a faster development occurred in embryos of the lobster *H. gammarus* when the eggs contained more triacylglycerides as an energy reserve. Clarke *et al.*, (1990) found that *M. rosenbergii* uses both triacylglycerides and phopholipids as energy sources during its embryonic development. In the present study the measurement of triacylglycerides is taken as a measurement of the energy content in the

embryos to analyse the differences of that parameter in the three different alkalinity levels where the embryos were exposed during their development.

4.1.3. Biochemical components in relation to embryonic energy

The development of the embryo is accompanied by an internal metabolic consumption of energy reserves throughout the whole series of morphological changes. Starting immediately after the fertilisation of the egg, this continues until the larvae hatches and the whole yolk sack is consumed (Balasundaram and Pandian, 1982). These energy and nutrient reserves are generally stored in the eggs as lipids and proteins (Wickins *et al.*, 1995).

4.1.3.1. Lipids

Lipids are biomolecules that are highly soluble in organic non-polar solvents such as chloroform, ether or benzene, and water-insoluble (Weete, 1980; Stryer, 1988). They have several biological functions: they serve as fuel molecules, highly concentrated energy stores, signal molecules, and components of membranes. The three major kinds of membrane lipids are phospholipids, glycolipids, and cholesterol (Table 19.). Cholesterol is important in the composition of membranes, because it is the moderator of the fluidity through them (Stryer, 1988).

Table 19. Membrane lipids.

Derivates of glycerol	Phosphatidyl choline
	Phosphatidyl ethanolamine
	Phosphatidyl serine
	Phosphatidyl inositol
	Phosphatidyl glycerol
Derivate of sphingosine	Sphingomyelin
Sugar-containig lipids	Glycolipids
Lipid	Cholesterol

4.1.3.1.1. **Fatty acids**

According to Stryer (1988), fatty acids have three major physiological functions:

- First, they are building blocks of phospholipids and glycolipids, which are important components of biological membranes.

- Second, fatty acid derivates serve as hormones and intracellular messengers.

- Third, they are fuel molecules, which are stored as triacylglycerols, also called neutral fats or triglycerides.

4.1.3.1.2. Triacylglycerols

Triacylglycerols, which are uncharged esters of glycerol, are highly concentrated energy stores of metabolic energy. A comparison between those molecules and carbohydrates and proteins would show that the energy of a complete oxidation of fatty acids is about 9 kcal g^{-1}, which is more than double for carbohydrates and proteins (about 4 kcal g^{-1}). The basis of that large difference in caloric yield is that fatty acids are more highly reduced than carbohydrates and proteins. Furthermore, triacylglycerols are very non-polar, therefore they are stored in a nearly anhydrous form, whereas proteins and carbohydrates are more polar and hence more highly hydrated (Stryer, 1988). The same author mentioned that a gram of dry glycogen binds about two grams of water, and consequently a gram of nearly anhydrous fat stores more than six times as much energy than a gram of hydrated glycogen, and concluded that that is the reason why triacylglycerols rather than glycerols were selected in evolution as the major energy reservoir.

4.1.4. Vitellogenic reserves for larval development and survival

Endogenous energy reserves from the yolk sack are used by many marine as well as fresh and brackish water species during the first few days of their development. An egg contains all the nutrients for the embryo to develop and hatch as a larva and even for this to reach its first feeding stage without trouble (Ling, 1969; Mourente and Vazquez, 1996). However, in migratory

species such as *M. rosenbergii*, there are two factors that could affect the subsequent development of the larvae. First, any environmental factor such strong tides, river currents, rain, and any obstacle that could stop the larvae from reaching the adequate salinity in a river-estuary system. Second, is the timing of hatching, Wickins and Beard (1974) found that *M. rosenbergii* hatch the entire egg mass in one or two batches. Similarly, Gomez Diaz and Ohno (1986) mentioned that this species could hatch in two batches in two consecutive days under suboptimal environmental temperature (25 °C.). *M. nobili* larvae usually hatch in batches between a period of 3 to 6 consecutive days (Pandian and Balasundaram, 1982; Balasundaram and Pandian, 1982). All the larvae hatch in the same stage of development, but those larvae, which hatch later on, have been consuming their embryonic energy reserves (Balasundaram and Pandian, 1982). Therefore, either by environmental obstacles in the journey or by a delay in the hatching time, if the larvae do not reach the site with the appropriate salinity (approximately 12 ‰) where it can moult into the second developmental stage to start feeding on the available food, those nutritive reserves could be depleted to a lethal extent or to affect the subsequent viability.

4.1.5. Larval development

Romdhane *et al.*, (1995) found that the larvae of *M. rosenbergii* requires a minimum of 35 mg g^{-1} of omega-3 highly unsaturated fatty acids in their diet. *Artemia* spp. is the source of food most commonly used in hatcheries

and laboratories of larval production. However, it is known that *Artemia* nauplii are deficient in essential polyunsaturated fatty acids (McEvoy and Sargent, 1998), therefore, an enrichment is added to help the nutrition of the larvae. Devresse *et al.*, (1990) mentioned that the larval time to reach metamorphosis could be reduced from 20-34 to 19-28 days by using enriched *Artemia* nauplii with w-3 fatty acids as food for the larvae. However, not only the diet has an influence in the development of the larvae and the time required for metamorphosis, but also environmental parameters and life history. Ling (1969) already mentioned that the larval development has a large variation among the organisms of a single batch after the third larval stage, and Uno and Kwon (1969) similarly reported that even in the same stage of development there is a high variation in the size of the larvae of the same batch from stage tenth. Gomez Diaz (1987) found that embryos incubated at 25 °C. produced larvae capable of metamorphosing in a wider range of temperatures (22 to 31 °C.), than those from embryos incubated at 29 and 31 °C. Funge-Smith (1991) found differences in the total body calcium concentration in the first larval stage of *M. rosenbergii*, from 790 to 980 mmol kg^{-1} (dry weight) when the larvae were reared in fresh and brackish water respectively, and he suggested that it could be due to the incubation media of the embryos (fresh or brackish water). Christiansen (1988) mentioned that the duration of the moulting cycle in larvae could be affected by exogenous and endogenous factors like temperature and regeneration of lost appendages. Therefore, the incubation media and the effect that this parameter could have on the embryos, could have an effect on the development of the larval stages of the species and their subsequent postlarvae and juvenile stages.

4.1.6. Postlarval development

As mentioned earlier, most of the work on the adaptability of crustacea to different environmental parameters has been done specifically with reference to salinity and temperature. Richard in 1978 found that postlarvae of *P. serratus* acclimatised to low temperatures (13 °C.) were more resistant to extreme low temperatures than the unacclimatised postlarvae, but no so to high temperatures. Stern *et al.*, (1984) acclimatised *M. rosenbergii* to different salinities and ion concentrations to measure the oxygen consumption and the ammonia production. Similarly, Sandifer *et al.*, (1975) acclimatised *M. rosenbergii* prawns to different salinity concentrations, for periods ranging from several days to five months, to evaluate the salinity tolerance of the postlarvae and juvenile stages. Singh (1980) mentioned that the osmoregulatory capacity of adult *M. rosenbergii* could be increased in a similar way as the one of postlarvae, by a gradual acclimation of the adults to different salinities. Stern *et al.*, (1987) suggested that the differences in the results of several studies in the osmoregulatory ability of *M. rosenbergii* are associated with the period that the prawns were acclimatised to the test media prior to the analyses in each report. Thus, acclimatisation of embryos of *M. rosenbergii* to high environmental alkalinity could increase the tolerance limits to that variable in latter developmental stages of the species.

4.2. METHODOLOGY

The experiment for the embryonic analysis was run at the same time as the experiment on the larval development, for logistical reasons relating to breeding of the prawns.

There were three different alkalinity concentrations (25, 100 and 200 mg L^{-1} as $CaCO_3$) in which three groups of six berried female prawns were kept. From these six females, three of them were kept without disturbance (in each treatment) to allow the production of the larvae used in the next experimental section. Each female prawn was sampled every five days in order to collect embryos at different stages of development for their later analysis of lipid content (specifically triacylglycerides (TAG)).

4.2.1. Experimental water recirculating systems

The three recirculation systems were built identically. Each system had a 1200 L h^{-1} pump with a maximum head of 2 m (403 Fluval). A filter of activated carbon (Eheim Standard filter carbon) was added to provide additional water treatment capability to the biological filters in each system (Forteath, 1993). The water from the header tanks flowed to the holding tanks by gravity through the main distribution lines (PVC pipes 2.5 cm in diameter) (Plate 14.). Each main distribution line was then divided in eight to supply all parts of each raceway. Those divisions were made down to a 3 mm diameter tubing for this

purpose with connections made of bored rubber bungs held in place with a plastic 1 mL pipette tubing (Sterilin T.D.). The water of the same alkalinity in each treatment was controlled with small manual plastic gang valves (Algarde) in order to provide the same flow (32.4 L h^{-1}) to each of the eight divisions.

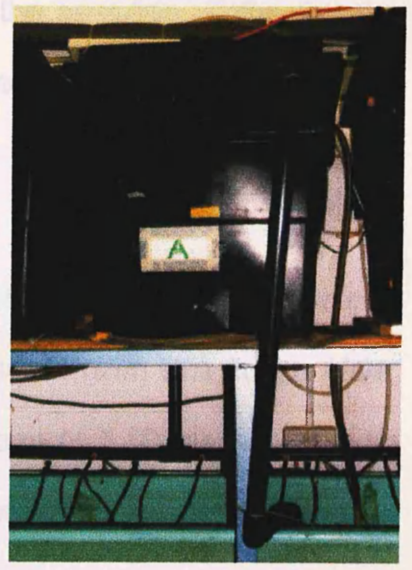


Plate 14. Header tank and water distribution line.

4.2.2. Selection of the broodstock

Although there are methods for identification of sexually mature females, like the one described by Sagi and Ra'anan (1985) where they used a dominant male to select the females that were in the pre-mating moult, the females used for these experiments were not selected according to that criteria. Instead females were selected as soon as they were mated and produced eggs. The size of the females (used for the sampling of the embryos) was in the range of 29.8 to 43 g and 28 to 39 mm carapace length (CL). Studies on the prawns held in individual tanks in the Tropical Prawn Unit and the Tropical Aquarium have shown that above 40 mm CL, prawns are no longer growing well probably due to restricted space but also possibly related to limitations of the water quality and diet (Brown J.H., personal communications, 1995; Melatunan, 1997). Below 20 mm CL they are not likely to be sexually mature (personal

observations). These two criteria were used for the selection of the females used for the experimental purpose. Limited choice of mature females meant that size variation was unavoidable.

The broodstock was selected from the Tropical Aquarium because of some technical problems in the system of the Tropical Prawn Unit during 1998, which resulted in sudden changes in the temperature (± 5 °C) as well as high fluctuations in the concentrations of $CaCO_3$ in the water of the broodstock.

4.2.3. Transferring the berried females

Once the females were mated they were kept for 24 h in the Tropical Aquarium to ensure that the eggs would remain attached to the pleopods after transferring the females to their respective treatment in the Tropical Prawn Unit. Although it is known that unfertilised eggs are released within 2 or 3 days following their attachment to the pleopods on the female (Ling, 1969; Wickins and Beard 1974; Ganeswaran, 1989), it was observed that if the female was transferred to a different water quality system (in this case, different alkalinity and total hardness) immediately after she was mated then, the apparently fertilised eggs[1] (observed under microscope, 40 X) were released within 1 or 2 days. Furthermore, if a female was carrying the eggs for a short period of time (1 to 8 h), and then was transferred, she was able to keep the eggs for as long

[1] Eggs with a plain orange or dark yellow coloration were always fertilised and they were able to develop into larvae, while some of the egg batches that were showing dark spots randomly distributed in the yolk of the eggs were always aborted by the female.

as five days and then she would release them (personal observations). The procedure to mate them was the same as the one described in section 2.2.2. The broodstock were not mated in the treatment media, since it was not known if the levels of alkalinity could have an effect on the quality of the sperm, prior to the fertilisation of the eggs, and the process of the mating itself.

4.2.4. Berried females stocking procedure

4.2.4.1. Prestocking measurements

Prior to the stocking of the berried females into the different alkalinity treatments, they were weighed in a preweighed dark plastic container (2 L capacity) half filled with the water of the respective treatment on a Mettler AJ 100 balance. The carapaces of the female prawns were measured from the back of the base of the eye to the posterior mid-dorsal margin of the carapace itself. After the first weighing of the female, a sample of embryos was removed for future analysis (see section 4.2.7. for details). Most of the egg batches were of a dark yellow or orange coloration in their first day of development and most of them were completely covering the four first pairs of pleopods. If any of the females mated had any pair of appendages amputated, or melanin spots were present on the exoskeleton, or the appearance of the rostrum was abnormal, just after they laid the eggs, they were rejected.

4.2.4.2. Stocking

After the prestocking measurements, each berried female was individually transferred into an incubating tank (Plate 15.), being a white translucent polypropylene container (0.34 m L* x 0.22 m W* x 0.15 m H*) covered with a lid (polycarbonate). Those containers were individually supplied with air and were under a 12:12 hours photoperiod. The female prawns were kept there for a period of fifteen days in their respective alkalinity treatments. They were kept without disturbance, except for embryonic sampling every five days.

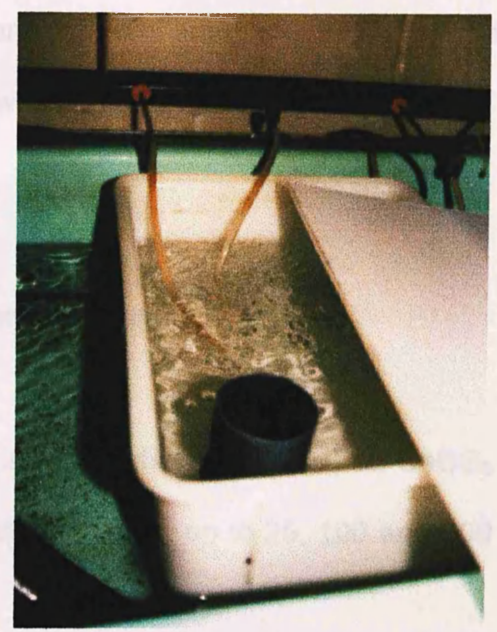


Plate 15. Embryonic incubation tank.

4.2.5. Management of the broodstock

4.2.5.1. Feeding regime

All the berried females were fed with the same food and the cleaning routine was the same as described in section 3.2.7., but the size particle of the food was larger depending on the size of the females.

4.2.5.2. Measurement of water quality parameters

Dissolved oxygen, salinity, temperature, total ammonia, alkalinity and total hardness were measured in the same way as described in section 2.6.

4.2.6. Alkalinity and total hardness experimental levels

Total hardness was kept at a constant level of 100 mg L^{-1} as $CaCO_3$ for all the different treatments, while alkalinity was made up to 25, 100 and 200 mg L^{-1} as $CaCO_3$.

4.2.7. Sampling of embryos

The embryos were sampled every fifth day from the first day that they were laid to the fifteenth day of development for each female prawn used. The sampling was carried out with the minimum possible disturbance of the female prawn. The lid of an individual incubating tank was partially removed to allow the introduction of a fine mesh in order to catch the female and to allow it to be manipulated and turned up side down (Plate 16.). Once the female was turned upside down to see the whole batch of eggs, a small amount of embryos (0.07 ± 0.03 g) was removed from the setae of the first pair of pleopods with a dissection forceps. Once the sample was removed from the female, she was

then put back into her tank and the lid was replaced in the original position to prevent her jumping out of the tank.

Plate 16. Female prawn ready to be sampled for embryos.

The sample of embryos was transferred to a transparent cristal polystyrene Petri dish (Merck), and then divided into two similar quantities into two new Petri dishes.

4.2.7.1. Storage of subsamples of embryos for triacylglycerides (TAG) analyses

The first subsample was weighed to the nearest 0.1 mg and then stored in a glass (Chromacol) vial of 2 mL capacity with a solvent resistant lid. Between 1.5 and 2 mL of a solution containing Chloroform and Methanol in a proportion of 2 : 1 and 0.01% of butylated hydroxytoluene (BHT), was added to the vial containing the subsample. After that, pure nitrogen gas (N_2) was introduced to the vial in order to diminish the oxygen at the surface of the

solution and therefore reduce the possibility of an oxidation of the lipids in the sample. In that way, the production of free fatty acids and a reduction of the total percentage of the TAG could be prevented. The subsample was then stored in a freezer set at minus 20 °C.

4.2.7.2. Description of the subsamples of the embryos.

The second subsample was weighed in the plastic dish and afterwards 1 mL of water, from the same tank where the female prawn was kept, was added to the plastic Petri dish, in order to prevent the eggs from drying up. The water was agitated for a few seconds to allow some embryos to separate from the rest. If a minimum number of 30 embryos were separated then they were measured, if not, the eggs were gently prised apart with the help of two needles, taking care in order not to break the thin external membrane of the embryos. Each one of the 30 embryos was measured in both longitudinal and transversal diameters under a micrometer (Graticules Ltd. 100 x 0.1 = 10 mm) installed in a compound microscope (1 X) (Olympus VMT). The formula to calculate the volume (Jeffrey, 1995) of an ellipse was used in order to know the volume of the embryos (Figure 11.).

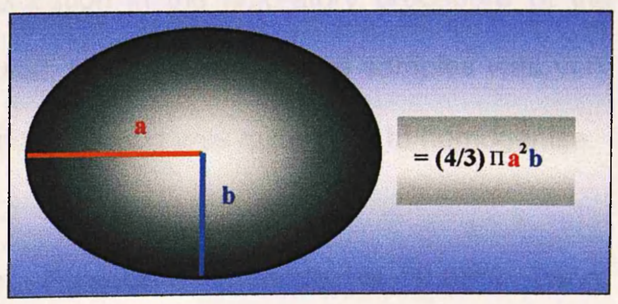


Figure 11. Volume of an ellipse.

A few drops (4 to 6) of sodium hypochlorite 12% concentrated were added to the Petri dish, to dissolve the interconnecting membrane between the eggs. Once the eggs were separated, they were transferred with a plastic Pasteur pipette to a Bogorov tray on a dark surface and counted with a counter tally.

The embryonic development was followed on every sampling day. Characteristics of the shape, colour, and new structures of the embryo were recorded (i.e. appearance of the eye spots and heart). The stage of the embryonic development was described according to Uno and Kwon (1969).

4.2.8. Embryonic energy content through Triacylglycerides analyses

4.2.8.1. Total lipid extraction

Once all the samples were collected, they were processed for their total lipid extraction. A slight modification in the laboratory procedure of the Folch *et al.*, (1957) method was used, since the size of the samples was very small (under 0.1 g of wet weight) and the tissue was quite easy to homogenise.

Three stock solutions, Potassium Chloride at 0.88% (w/v), Chloroform / Methanol 2 : 1 (v / v) and Chloroform / Methanol 2 : 1 (v / v) +

butylated hydroxytoluene (BHT) 0.01%, were prepared before homogenising the tissues.

The tissue of each sample was homogenised in 10 volumes of Chloroform / Methanol 2 : 1 (v / v) in a (Pyrex) glass / Teflon homogeniser (Glas-Col Terre-haute 47802) keeping the sample surrounded by ice in order to prevent temperature rise and to avoid vaporisation of the solvents. After the homogenisation of the tissue, the resulting solution was filtered using a funnel (Pyrex) and a prewashed filter paper (Whatman No. 1) into a test tube (Pyrex, 15 mL capacity). Potassium Chloride 0.88% (w / v) was then added to the filtered solution in a proportion of 8 : 4 : 3 of Chloroform : Methanol : Potassium Chloride 0.88%. The solution was vigorously mixed and then centrifuged at 1500 rpm in a MSE centrifuge. After that, the solution developed two phases of different density, the organic phase (solvents and lipids) were in the bottom of the test tubes and the aqueous phase in the top, which was then removed with a glass pipette (Volac 150 mm) connected to a vacuum system. The organic solution was refiltered as before and then evaporated to dryness under nitrogen (N_2) gas on an analytical evaporator (N-EVAP). After the lipids were totally dry, they were redissolved with less than 1.5 mL of Chloroform / Methanol 2:1 and transferred to a preweighted glass (Chromacol) vial. The solution was redried in nitrogen (N_2) gas as before and then it was desiccated overnight in a borosilicate glass vacuum desiccator (Dry-Seal). The weight of the total lipids was then calculated and a concentration of 10 mg of total lipid per mL of Chloroform / Methanol + BHT was prepared in the same vial and flashed up

with nitrogen (N_2) gas. This solution was stored at minus 20 °C for further lipid classes analysis and determination of triacylglycerides (TAG).

All the equipment used for the extraction of lipids was made of glass because the two solvents used are corrosive to plastics and corroded plastics could contaminate the samples. All the chemicals used for the lipid extraction were of high purity reagent grade, for HPLC applications.

4.2.8.2. Analysis of lipid classes by HPTLC

Double development method using High Performance Thin Layer Chromatography (HPTLC) 10 x 10 cm plates of silica gel 60 (Merck) was used in order to analyse the lipid classes (Olsen and Henderson, 1989) (see Appendix IV,a). The graphical analyses of the lipid classes were done in a Shimadzu Dual-Wavelength Flying-spot Scanner CS 900. TAG was estimated as a percentage of the total lipids of each sample and it was compared between the three different treatments and through the experimental sampling times.

4.2.9. Larval rearing procedure

4.2.9.1. Broodstock selection and management for larval experiment

The protocol followed in this experimental section for the selection and management of the broodstock was the same as the one described for the female prawns used for the incubation of the embryos from section 4.2.2. to 4.2.6. Nine females were selected in order to produce three groups of progenitors and larvae for each treatment (alkalinity of 25, 100 and 200 (mg L^{-1} as $CaCO_3$). The range in weight and carapace length of the female prawns was between 25.8 to 35.7 g and 23 to 31.4 mm respectively. The broodstock was distributed in a way that every treatment had a small, medium and large female prawn.

4.2.9.2. Larvae hatching operation

Once the embryos were in their 15 to 20th day of the incubation period and development, the larvae hatched in the same system as described in section 2.2.3.

4.2.9.3. Monitoring the development of the larvae

This experiment was carried out with the larvae hatched from the embryos previously held at the different levels of alkalinity (25, 100 and 200 mg L^{-1} as $CaCO_3$). The larvae from the nine females were reared in quadruplicate. Each group of larvae equivalent to one replica was made up by 100 larvae.

4.2.9.4. Experimental system design

All the larval rearing containers (LRC) were made out of transparent plastic bottles (Coca Cola 2 L plastic bottles). Those containers were allocated in a clear water recirculation system (Plate 17.). The recirculation system was as described in section 2.1.3., but there were two 250 L polypropylene black tanks (Wizard) for biological filtration. The header tank contained an electrical submersible heater (1500

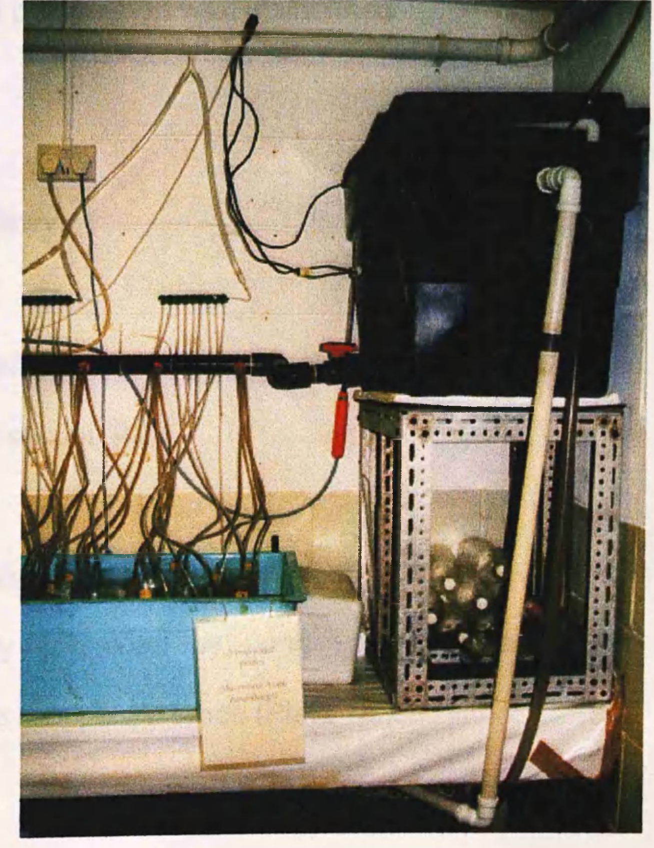


Plate 17. Recirculation system for experimental larval rearing.

Watts thermostatically controlled heater Galvatek) to keep a water temperature of 29 ± 0.5 °C. The raceway was fitted with holders made from PVC tube (11 cm in diameter by 14 cm high) to keep the containers (LRC) for each group of larvae. A nylon mesh filter was adapted at the exit of the drain of the raceway in order to collect any suspended solids from the water column. The inlet water supply was from the header tank and was fed by gravity into a main distribution line (2.5 cm (internal diameter) PVC pipe) and then distributed to each container through reduced 3mm plastic lines. The water flow in each different container (LRC) was the same, being 14.7 L h^{-1}, and that was controlled by a valve in the main distribution line. In one of the biological filter tanks, at the bottom of the system, there was a submersible pump (Eheim 1060, 240 volts) which was pumping the water to the header tank after being passed through the biological and mesh filters. A 12 : 12 hours photoperiod was maintained.

4.2.9.5. Water Chemistry

Temperature, total ammonia, pH, and dissolved oxygen, were measured in the same way as in section 2.6.

Aeration was supplied by the main distribution line in the Tropical Prawn Unit and was manually controlled by gang valves (Algarde) and distributed to each container by 3 mm plastic tubes (Plate 18.).

The salinity of the water of this system was kept at 12 ‰ by a mixture of fresh (dechlorinated) tap water and full sea water. Preheated fresh tap water from a reservoir tank was added to the system every day, in order to compensate evaporation losses, which were in an order of 5 to 10 L in the 800 L brackish water system.

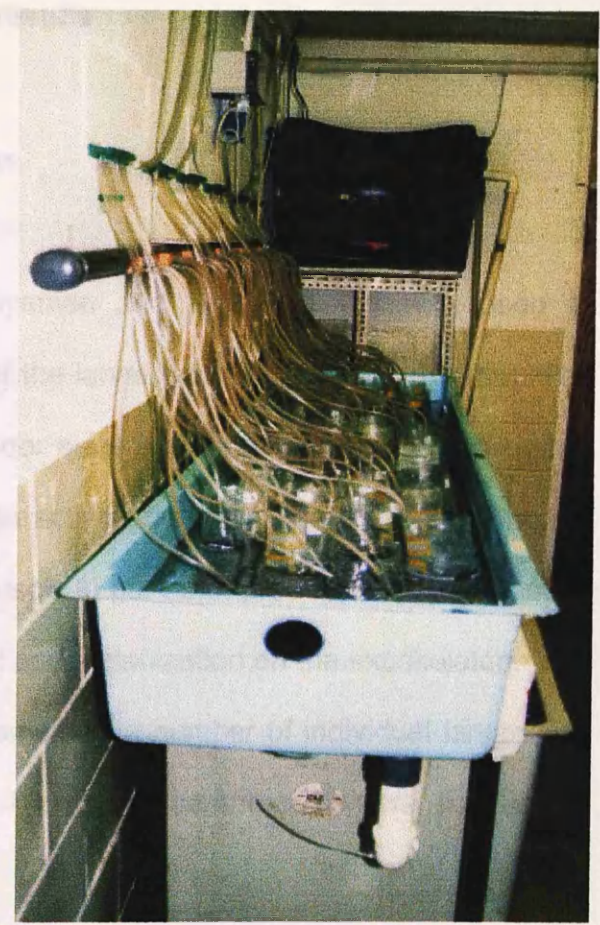


Plate 18. Air distribution to the LRC.

4.2.9.6. Management

Enriched *Artemia* metanauplii (approximately 48 ± 2 hours from hatch) were prepared on a daily basis and were fed to the larvae from the second day of their development and all the way through to the metamorphosis stage.

4.2.9.7. Presentation of results

4.2.9.7.1. Condition index

The condition index (Tayamen and Brown, 1999) was used to estimate the general health status of the larval group (see Appendix V,a). Ten larvae were monitored every day under a stereoscopic microscope and scored for a number of features such as: their activity, the state of the chromatophores, coloration of the whole organism, muscle to gut ratio, presence of lipid drops in the anterior part of the cephalothorax and melanization on the exoskeleton. This survey allowed a quantitative assessment of a number of individual larvae and allowed comparisons to be made between different containers (LRC) and different treatments.

4.2.9.7.1.1. Modified parameters in the condition index

A modification in the way of assessing the larval quality was done after the first four days of observations and comparisons of the larvae, due to subjectivity of the index itself. A narrowed selection of factors was used to establish the quality of the sampled group of larvae, since some of the factors of the larval condition index were not applicable for this study.

4.2.9.7.1.1.1. Gut fullness

The fullness of the gut was a variable factor depending on the time of sampling. According to the time of observation, the gut was full or empty, but in the next minute it could be the opposite, due to the digestive activity and the peristaltic movement of the gut itself. It was observed that during the sampling time, some organisms were egesting some faeces and therefore the gut was being filled and emptied in a very short period of time, even though when the sampling time was fixed to be 1 hours after feeding. If the organism was with an empty gut, the score index was negatively skewed. Furthermore, the sampling time was quite long due to the number of treatments and replicates, (between 1 and 2 hours) and if the changes in the gut appearance were noticed to occur in a matter of minutes, the possibility of sampling the larvae in the wrong time was increased. Therefore, this parameter was not used for the evaluation of the larvae quality.

4.2.9.7.1.1.2. Gut lipid content

Gut lipid content (state of hepatopancreas) was scored only in two categories and not three as specified in the index. The reason for this was that although some larvae were growing faster than others, all of them have some lipid vacuoles at the time of sampling. This could be due to the *ad libitum* supply of *Artemia* spp. in the larval containers, giving enough source of lipid to each one of the larvae.

4.2.9.7.1.1.3. Muscle appearance of abdomen

The abdominal muscle appearance was subject to light intensity and therefore it was not considered in this study. If the microscope was adjusted to see through the three-dimensional organisms then, the effect of the light changed the transparency of the abdominal muscle itself, giving a sort of corrugated appearance. Furthermore, the exoskeleton had an effect on the opacity or transparency of the muscle.

4.2.9.7.1.1.4. Fouling organisms

None of the larvae sampled had any fouling organisms. Therefore, this parameter was ignored in order not to overvalue the quality of the larvae. Similarly in Funge-Smith (1991), the fouling organisms were almost completely eradicated from his experiments, when the use of biological filters was added to the systems. Wickins (1976), said that in closed systems, the management of the system has more control over the introduction of diseases, for example the food supplied is made of clean and sterile ingredients. Furthermore, the water in the Prawn Unit is always filtered and changed periodically and the experimental system is entirely cleaned in a regular basis. That makes it more difficult for protozoans or parasites to infest the animals, besides which, the benign colonies of bacteria in the system compete with possible external infestations.

4.2.9.7.2. Developmental stages

The larvae were also observed under a stereoscopic microscope, in order to record each stage of development and the time consumed to grow to the next stage. The larval stages were described according to the guide developed in 1969 by Uno and Kwon (see Appendix V,b). However a brief description of the different developmental stages and their cardinal characteristic is shown in Table 20.

Table 20. Main features of larval developmental stages of *M. rosenbergii*.

Stage of development	Main feature
I	Sessile eyes
II	Stalked eyes
III	Uropods appear
IV	Second spine in the rostrum
V	Uropods biramous separate from telson
VI	Buds of pleopds appear
VII	Pleopods biramous and bare
VIII	First setae on exopodite
IX	Pleopods covered by setae
X	First three dorsal teeth on rostrum
XI	Dorsal rostrum totally toothed
Postlarvae	Rheotaxis

4.2.9.7.3. Survival

The survival of the larvae and all newly developed postlarvae were recorded every day as well as the time taken for them to reach the

metamorphosis stage, in order to estimate the average time period to reach the metamorphosis stage for each of the larval groups. This was done to observe the differences in the different groups of larvae, reared under the same physico-chemical conditions and feed with the same enriched *Artemia* spp. but being offspring of different embryonic groups incubated at different levels of alkalinity.

4.2.10. Preconditioning of the new postlarvae to freshwater

Once the larvae had metamorphosed, they were gradually conditioned from brackish to freshwater (12 ‰ to 0 ‰) in a 24 hours period. This procedure was done in a separate recirculating system (similar to that of the larval rearing system) with a salinity of 6 ‰ and a temperature of 29 ± 0.5 °C. The newly metamorphosed postlarvae were collected from the four replicate (LRC containers) of the same group of larvae, in a daily basis, and pooled together to complete (by triplicate) 10 organisms to be transferred into a nylon mesh pot (18 cm in diameter by 18 cm in high) (Plate 19.). Each mesh pot contained a shelter of two black PVC perforated plates, in order to increase the substrate surface for the 10 postlarvae inside the mesh pot. These mesh pots were kept into the 6 ‰ brackish recirculation water system. The postlarvae were left there for a period of 24 hours minimum and until a total of 10 postlarvae were collected for each

Plate 19. Mesh pot for postlarval rearing.

mesh pot from the four LRC containers. They were fed *ad libitum* with tropical fish flakes (Aquarian).

4.2.11. Measurements of the postlarvae

Once the 10 postlarvae were in the mesh pot for a minimum period of 24 hours in the 6 ‰ salinity system, they were collected and gently dried on soft absorbent tissue in order to weigh them. The same protocol as in section 3.2.3. was applied to dry and weigh the new postlarvae.

4.2.12. Transferring the postlarvae to different alkalinity waters

After acclimation over 24 hours to freshwater postlarvae were transferred to one of the three different alkalinity levels (25, 100 and 200 mg L^{-1} as $CaCO_3$). Postlarvae from one female were divided between the three alkalinity levels. This was done to reduce the variability within the three replicates and within the treatments.

4.2.13. Water Physico-chemical parameters

All the groups of postlarvae were kept in the same physico-chemical conditions except for the alkalinity levels in the water and all the water physico-

chemical parameters were the same as the ones described in sections 4.2.5.2. and 4.2.6. for the water system of the broodstock.

The water flow was the same as described in section 4.2.1. for the broodstock system, but in this case the 8 inlets were discharging directly into the raceways where the mesh pots were lying. Therefore the water change was through the walls of the mesh pot and so there was no disturbance of the postlarvae neither from the water inlet nor from the aeration of the water.

4.2.14. Management of the postlarvae

The diet given to the postlarvae was exactly the same as the one given to the berried females (section 4.2.5.1.), in both cases it was supplied *ad libitum* twice during the day, keeping an 8 hours period between the first feeding time and the second one. The only difference was the size, the pieces were smaller for the postlarvae than those fed to the broodstock. The uneaten food was removed every time before the next feeding took place by siphoning the bottom of the mesh pots and the raceways were siphoned twice per week, since only very few small particles that escaped from the mesh pots were there.

4.2.15. Growth rate and survival monitoring of the postlarvae

The postlarvae were weighed as a population and the biomass was recorded once every 7 days, this procedure was repeated during 8 consecutive weeks. At the end of that period, the postlarvae were weighed both as a group and individually. The survival of the postlarvae was monitored every day.

4.3. RESULTS

4.3.1. Physico-chemical parameters

The variations of the environmental parameters of the embryonic incubation water system are shown in Table 21.

Table 21. Physicochemical parameters in the embryonic acclimatisation water system.

Alkalinity *		Total hardness *		DO_2	Temp.	pH	NH_4-N
Req.	Obt.	Req.	Obt.	(%)	(°C.)		(mg L^{-1})
25	24.5 ± 2.1	100	102.3 ± 4.2	86.8 ± 4.6	29 ± 0.1	7.7 ± 0.1	< 0.2
100	97.1 ± 2.5	100	102.9 ± 3.7	84.5 ± 4.2	28.8 ± 0.2	8.4	< 0.2
200	200.6 ± 3.2	100	100.3 ± 3.8	84.8 ± 3.6	28.7 ± 0.1	8.7	< 0.2

* = measured in (mg L^{-1} as $CaCO_3$); Req. = Required; Obt.= Obtained; DO_2 = Dissolved Oxygen; Temp. = Temperature; NH_4-N = Total ammonia nitrogen.

4.3.2. Embryonic development

Day 0 was considered to be 24 hours after the female was mated, in order to allow the eggs to be completely attached to the pleopods of the female and, therefore, able to be transferred to the incubation media. The initial weight of the newly fertilised eggs was 80.8 ± 11.9, 81.2 ± 6.9 and 81.9 ± 5.4 μg in each of the alkalinity acclimatisation treatments at 25, 100 and 200 mg L^{-1} as $CaCO_3$ respectively.

4.3.2.1. Embryonic volume increment

The size of the eggs had a significant increment ($P < 0.05$) from the first sampling day to day fifteen (prior to the hatch of the larvae). However, that egg size increment was not affected by the different alkalinity treatments ($P > 0.05$). The embryos increased in a 39.5%, 40.8% and 39.2% of their initial size, and their final weight was 129.7 ± 10.1, 129.3 ± 23.6 and 119 ± 3.2 μg for alkalinity concentrations of 25, 100, and 200 (mg L^{-1} as $CaCO_3$) respectively. The final weight was not significant different ($P > 0.05$) between the embryos incubated at the different alkalinity and the volume increment of the embryos was very similar in all the sampling days (Figure 12.) for all the different treatments of alkalinity and no significant differences ($P > 0.05$) were found at any sampling time between the three treatments.

A general linear model for two factors was applied to find the statistical differences. The mean values were very close one to each other and their standard deviations were overlapping themselves, so therefore they were not plotted in this figure.

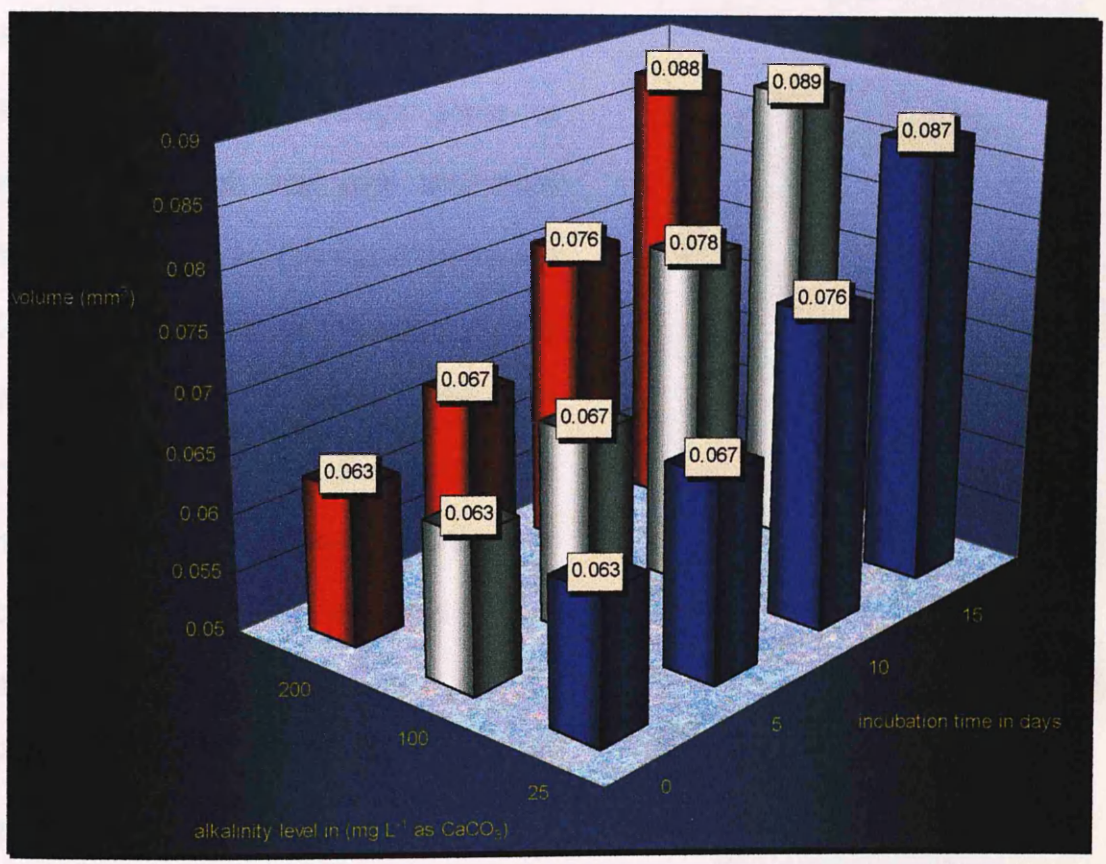


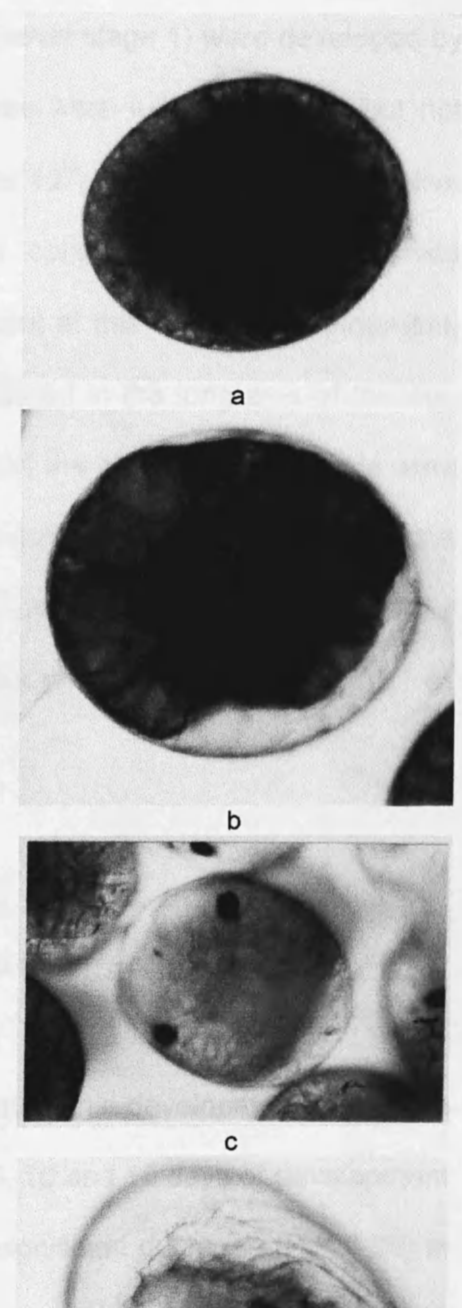
Figure 12. Volume increment every five days periods for embryos of *M. rosenbergii* incubated at different alkalinity levels.

4.3.2.2. Embryonic developmental stages

Plate 20. shows the different developmental stages of the embryos at the different sampling times (0, 5, 10 and 15 days). All the embryos were at the same developmental stage when they were sampled at days 0, 5, 10 and 15,

regardless of the alkalinity level in the incubation media. In Plate 20. “a” it is shown the full oval fertilised egg with a dark-yellow / orange coloration; this shape and colour was almost the same in every embryo at the beginning of the experimental time, although some of them were a bit less oval and more spherical, however, this could be related to the manipulation of the eggs (cutting and pulling the elastic inter-eggs membrane) to observe them under the microscope. A total “segmentation” or cell division pattern is described for this stage of development by Ling (1969). Plate 20. “b” shows a reduction in the yolk and a concentration of some cells (day 5th) to form the caudal papilla described by Ling (1969) at day 6 of embryonic development.

The eye pigment was developed and the heart was beating for all the embryos at the different treatments by day 10th (Plate 20. “c”).



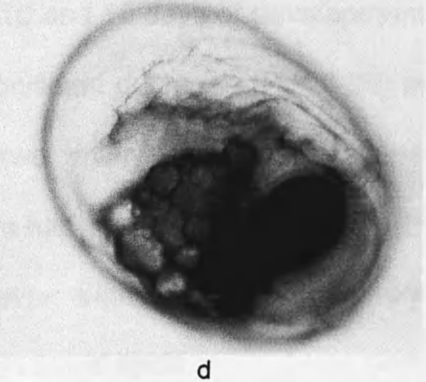


Plate 20. Different development stages of the embryos at the different sampling times (0, 5, 10 and 15 days for a,b,c and d respectively).

Most of the organs of the pre-hatched nauplii (larval stage 1) were developed by day 15th (Plate 20. "d"). By that time, the eyes were fully developed, but not separated, and the heart was larger than in day 10th; the vicinity of the digestive gland / stomach contained a few oily spheres (considered to be yolk reserves for the larval development). The gut was present at this stage of development, and most of the appendages were accommodated in the long axis of the egg and in some embryos they were vibrating, whilst the abdominal segments were bent in one focus of the egg. The times for the embryonic development until the larvae hatched did not show any significant differences ($P > 0.05$) being 17.7 ± 0.8, 17 and 17.8 ± 0.5 days for alkalinity levels of 25, 100 and 200 mg L^{-1} as $CaCO_3$ respectively.

4.3.3. Embryonic lipid classes and energy content

The lipid classes analysis for the embryonic development during the incubation period is shown in Table 22. for 0, 5, 10 and 15 days of development at each different alkalinity level. There was a significant decrease ($P < 0.05$) in the percentage of the neutral lipids. Its two main components were triacylglycerol and sterol esters, and both of them had a significant decrease ($P < 0.05$) at the end of the incubation period. However, alkalinity did not have any significant effect ($P > 0.5$) on the content of those neutral lipids.

Table 22. Lipid class composition (expressed as percentage of the total lipids) of embryos of *M. rosenbergii* at three different alkalinity levels.

Lipid class	Incubation Day	Alkalinity (mg L^{-1} as $CaCO_3$)					
		25		100		200	
		Mean (n=4)	Standard deviation	Mean (n=4)	Standard deviation	Mean (n=4)	Standard deviation
Sphingomyelin	0	0.2	0.1	0.2	0.1	0.2	0.1
	5	0.2	0.0	0.4	0.2	0.3	0.1
	10	0.5	0.1	0.6	0.3	0.5	0.2
	15	0.8	0.2	0.9	0.2	0.7	0.2
Phosphatidylcholine	0	14.7	1.2	14.6	3.1	16.0	0.4
	5	13.9	0.5	14.5	2.4	14.8	0.6
	10	13.0	1.3	14.1	1.8	13.3	0.9
	15	14.0	0.4	14.4	0.8	13.7	1.4
Phosphatdylserine	0	0.3	0.1	0.3	0.1	0.3	0.0
	5	0.5	0.1	0.5	0.1	0.5	0.0
	10	1.0	0.2	1.0	0.2	0.9	0.1
	15	2.0	0.2	1.7	0.4	1.8	0.2
Phosphatidylinositol	0	1.2	0.1	1.2	0.6	1.2	0.2
	5	1.4	0.1	1.5	0.1	1.4	0.1
	10	1.4	0.2	1.6	0.1	1.4	0.2
	15	1.7	0.1	1.7	0.2	1.6	0.2
Cardiolipin or Phosphatidylglycerol or Phosphatidic acid	0	0.0	0.1	0.2	0.2	0.1	0.1
	5	0.0	0.1	0.2	0.0	0.2	0.1
	10	0.2	0.0	0.4	0.2	0.2	0.1
	15	0.6	0.2	0.6	0.3	0.5	0.2
Phospatidylethanolamine	0	7.7	0.3	8.2	0.5	8.0	0.5
	5	7.8	0.6	8.2	0.3	7.7	0.4
	10	7.8	0.8	8.2	0.6	7.3	0.2
	15	9.1	0.4	9.3	0.9	8.8	0.3
Pigment	0	1.1	0.4	0.7	0.2	0.7	0.1
	5	0.7	0.2	0.8	0.1	0.7	0.1
	10	0.7	0.1	0.6	0.2	0.6	0.1
	15	0.8	0.2	0.9	0.2	0.7	0.1
Cholesterol	0	6.7	0.7	6.1	0.6	5.8	0.8
	5	6.6	0.8	6.2	0.7	5.8	0.9
	10	8.1	0.8	7.3	0.9	7.5	1.2
	15	10.8	1.3	9.4	0.5	10.6	0.4
Free fatty acids	0	0.7	0.6	0.4	0.2	0.2	0.3
	5	0.5	0.2	0.4	0.3	0.2	0.2
	10	0.7	0.1	0.6	0.2	0.4	0.2
	15	0.8	0.3	0.8	0.2	0.5	0.4
Triacylglycerol	0	57.6	2.2	59.3	2.7	59.1	1.4
	5	58.1	0.7	58.1	1.8	58.6	1.4
	10	58.1	2.0	56.5	1.9	58.4	1.8
	15	53.0	1.6	53.1	1.6	54.1	2.3
Sterol esters	0	9.9	0.6	8.8	1.2	8.4	0.9
	5	10.3	0.6	9.2	1.5	9.8	1.4
	10	8.4	0.8	9.1	2.2	9.3	1.5
	15	6.3	0.7	7.0	2.0	7.1	1.1
Total	0	100	0	100	0	100	0
	5	100	0	100	0	100	0
	10	100	0	100	0	100	0
	15	100	0	100	0	100	0
Total polar	0	24.1	1.5	24.6	4.2	25.8	1.0
	5	23.9	1.0	25.3	2.8	24.9	1.2
	10	23.8	2.5	25.9	2.6	23.7	1.6
	15	28.2	1.3	28.7	2.7	27.0	2.3
Total neutral	0	75.9	1.5	75.4	4.2	74.2	1.0
	5	76.1	1.0	74.7	2.8	75.1	1.2
	10	76.2	2.5	74.1	2.6	76.3	1.6
	15	71.8	1.3	71.3	2.7	73.0	2.3

The third major component of the neutral lipids was cholesterol and this increased significantly ($P < 0.05$) during the development of the embryo as well as trace amounts of free fatty acids for the last sampling period. Pigments did not shown any significant difference ($P > 0.05$) or a trend during the incubation period.

Opposite to the total neutral lipids, the total polar lipids had a significant increase ($P < 0.05$) over time, as the embryos developed when they were incubated at alkalinity levels of 25 and 100 mg L^{-1} as $CaCO_3$, and a strong tendency to increase was found when the embryos were incubated at alkalinity 200, but this was not significantly different ($P > 0.05$).

There were no significant differences in the changes of the total polar lipids regarding the alkalinity level in which the embryos were incubated, therefore just a sample of embryos from the treatment with 25 alkalinity was put in Plate 21. to show the proportional changes within the polar lipids themselves in a thin layer chromatography plate, during the four sampling days period.

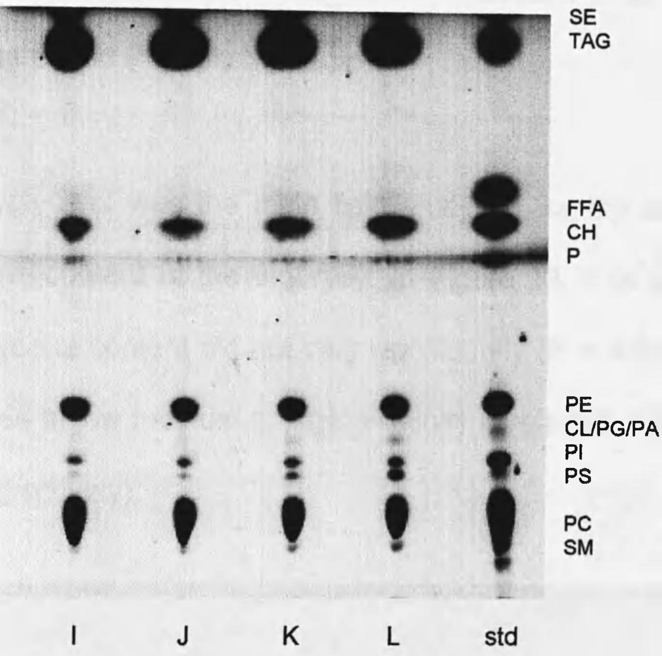


Plate 21. Sequential development of lipid classes through 15 days of development of an embryo of *M. rosenbergii*.
Each letter in the "X" axes corresponds to a group of embryos from the same female but in a different incubation time; I = day 0, J = day 5, K = day 10, L = day 15 and std = standard. The abbreviations in the "Y" axis correspond to: SE = Sterol esters, TAG = Triacylglycerol, FFA = Free fatty acids, CH = Cholesterol, P = Pigment, PE = Phospatidylethanolamine, CL/PG/PA = a combined fraction of Cardiolipin, Phosphatidylglycerol and Phosphatidic acid, PI = Phosphatidylinositol, PS = Phosphatdylserine, PC = Phosphatidylcholine and SM = Sphingomyelin.

The two main components of the polar lipids were Phosphatidylcholine and Phospatidylethanolamine, the latest had a significant increment ($P < 0.05$) during the developing of the embryo at the last sampling day, which is inverse to the triacylglycerols percentage. Phosphatidylcholine with the higher percentage of the polar lipids, however, had no significant variation ($P > 0.05$) but it developed a trend to decrease in the beginning of the embryonic development. Sphingomyelin had a significant increase ($P < 0.05$) in its percentage of the total polar lipids as well as Phosphatdylserine,

Phosphatidylinositol and the combined fraction of Cardiolipin, Phosphatidylglycerol and Phosphatidic acid.

Triaclyglycerol was the main lipid analysed for the consumption of energy in the development of the embryos. In Figure 13. it is shown that the average triacylglycerol content did not vary significantly (P > 0.05) between the embryos incubated at low medium or high alkalinity levels (25, 100 and 200 mg L^{-1} as $CaCO_3$ respectively).

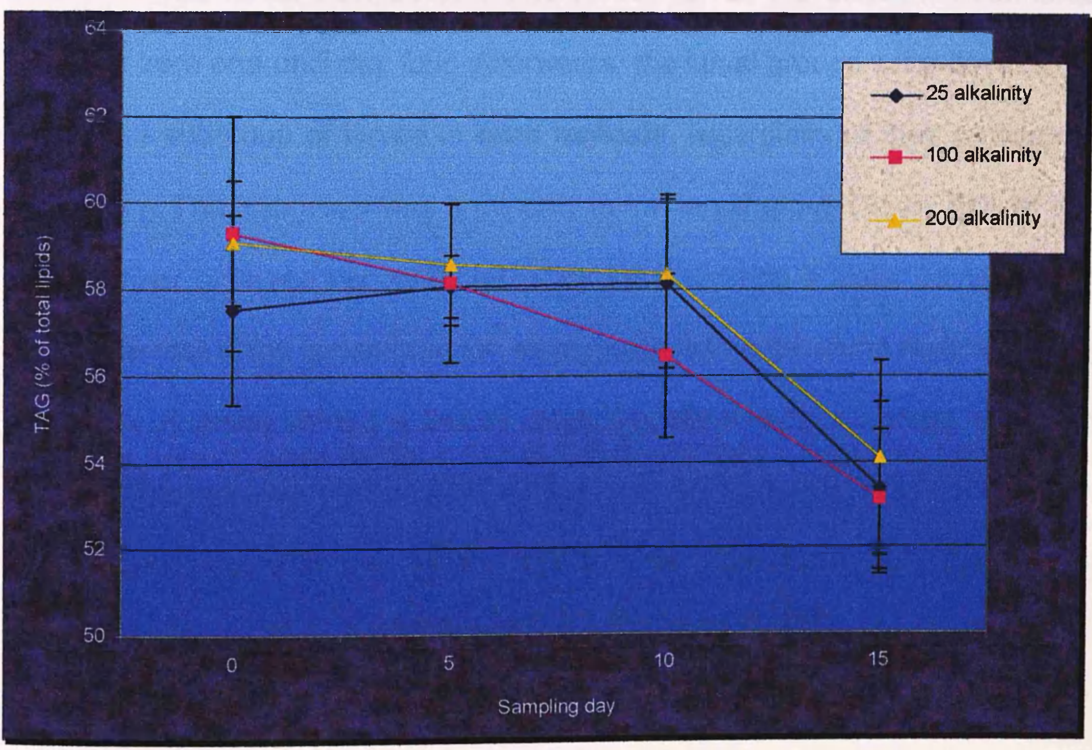


Figure 13. Triacylglycerol (TAG) content as percentage of total lipids in the embryos of *M. rosenbergii* incubated at different alkalinity levels in mg L^{-1} as $CaCO_3$.

However, the amount of triacylglycerol was significantly decreased (P < 0.05) as the embryo was developing from day 0 to day 15, regardless of the alkalinity levels in the incubation media. There was a trend to increase of the

triacylglycerol content for the first 5 days of the embryonic development, when they were incubated at low alkalinity levels, though the statistical analysis did not show any significant difference. A similar pattern was found by Clarke *et al.*, (1990) but they could also not give a good explanation for it.

4.3.4. Score Index for larval development

The larval development was similar for all the different treatments from day zero and until day four. Afterwards, the larval groups were developing a different subgroup of larvae in each replicate, regardless of their embryonic life history. This subgroup was a minority in the larval group (15 to 20% of the total number of larvae per replicate). They were showing a faster development above the rest of the larvae from the same batch and in the same replicate. This subgroup of larvae were the first to reach the metamorphosis stage and they were always more active than the rest. However as the larvae developed, some of the lower scored larvae moulted into the next developmental stage with a better score level. That pattern was the same until the larvae reached stage IX, X, XI and finally the postlarval stage, where they were highly scored as healthy organisms.

The average condition index for the larvae hatched from embryos incubated at different alkalinity levels did not show any significant difference ($P > 0.05$), and they were always above score 1 (Figure 14.).

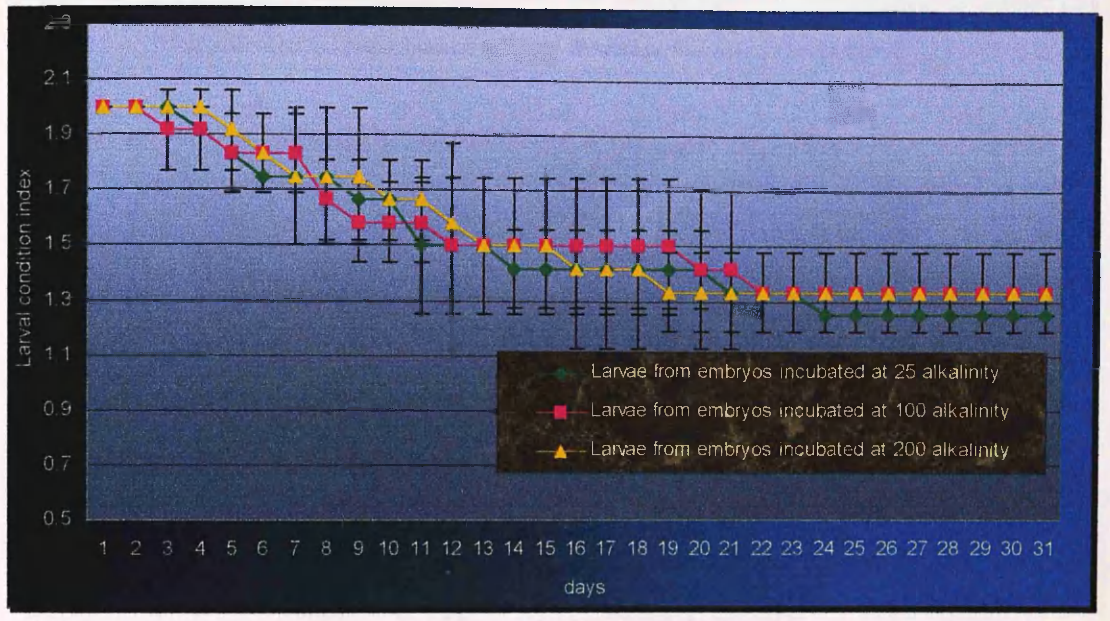


Figure 14. Scoring index for the development of *M. rosenbergii* larvae hatched from embryos incubated at different alkalinity (mg L^{-1} as $CaCO_3$) levels.

However, that condition index showed that the bulk of the larvae in the three different treatments were decreasing their quality as their development progressed from one stage to the next.

A significant difference was found ($P < 0.05$) when the condition index of the faster growing subgroup of larvae was compared with the average condition index of the rest of the larvae in each treatment (Figure 15.).

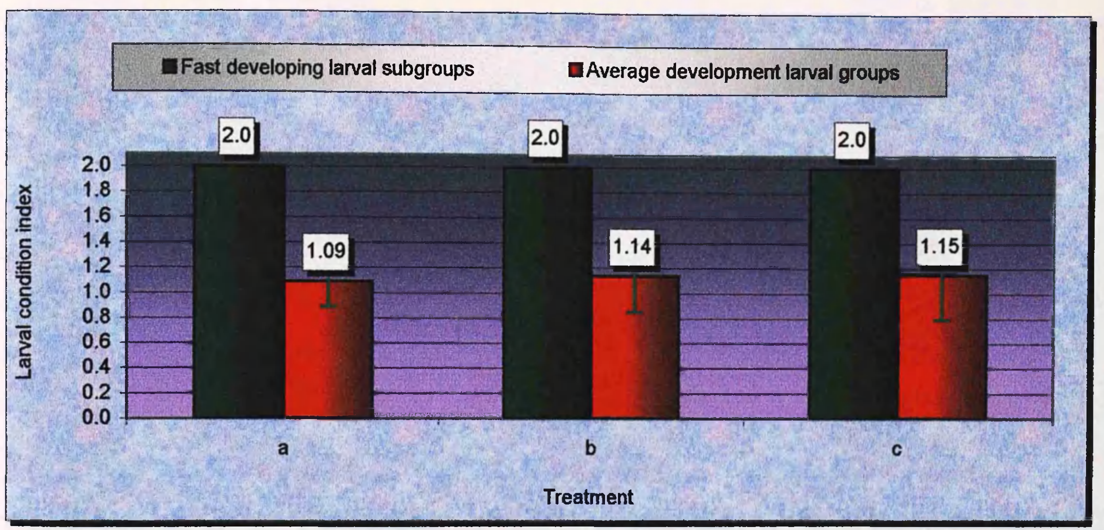


Figure 15. Mean larval score index for the subgroups of fast growing *M. rosenbergii* larvae and the rest of the larval groups.
Treatments a, b and c correspond to larvae hatched from embryos acclimatised at 25, 100 and 200 alkalinity (mg L^{-1} as $CaCO_3$) respectively.

4.3.5. Larval developmental stages

The values shown in Figure 16., are the mean values of the larval developmental stages plus their standard deviations of the healthy group of larvae in all the replicates for the different treatments.

There it is shown that the levels of alkalinity during the embryonic incubation period did not affect (P > 0.05) the development of the larvae in terms of the developmental stage reached each day up to the metamorphosis into postlarvae.

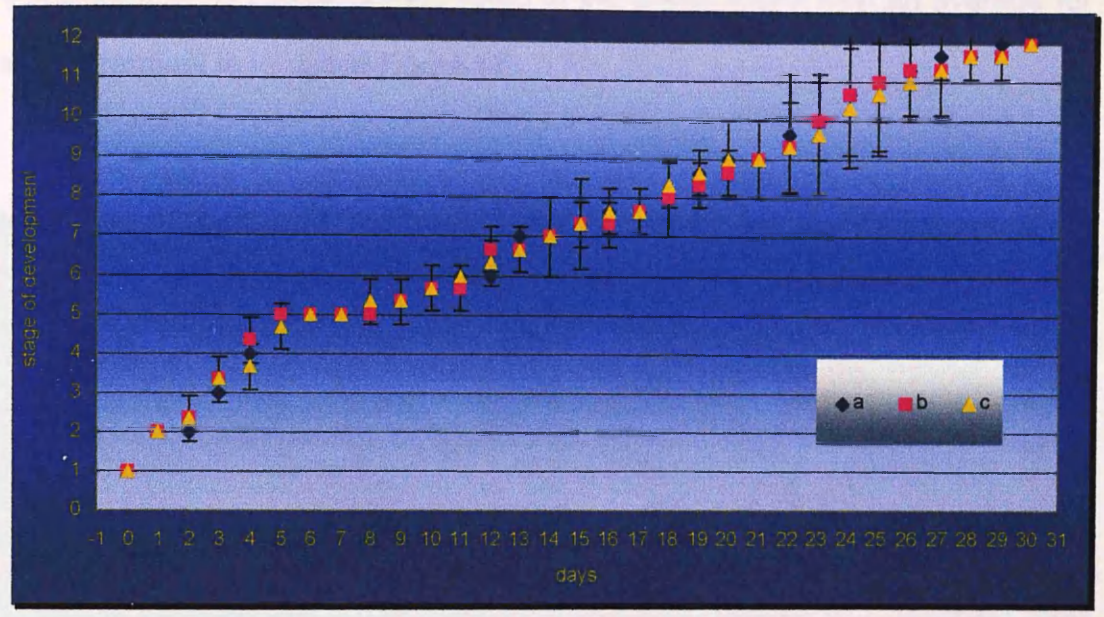


Figure 16. Sequence of developmental stages of the larvae of *M. rosenbergii*. Treatments a, b and c correspond to larvae hatched from embryos incubated at alkalinity levels of 25, 100 and 200 (mg L^{-1} as $CaCO_3$) respectively.

However, interestingly enough there is shown a major delay in the sequence of the developmental stages between the day 4 and 11. Between those days, the development of the larvae was stuck in stage five with little variation up to day 5 and after day 8, but days 6 and 7 did not show any variation at all. Another less marked delay is shown at stage eight, where the larvae were stuck from day 14 to day 23, for this period of time the variability was bigger.

4.3.6. Larval metamorphosis

The first larvae to reach the metamorphosis stage in each treatment were recorded individually regardless of the replicate number, and although the

first experimental postlarvae appeared at the twenty fourth day, an average for each treatment is shown in Figure 17.

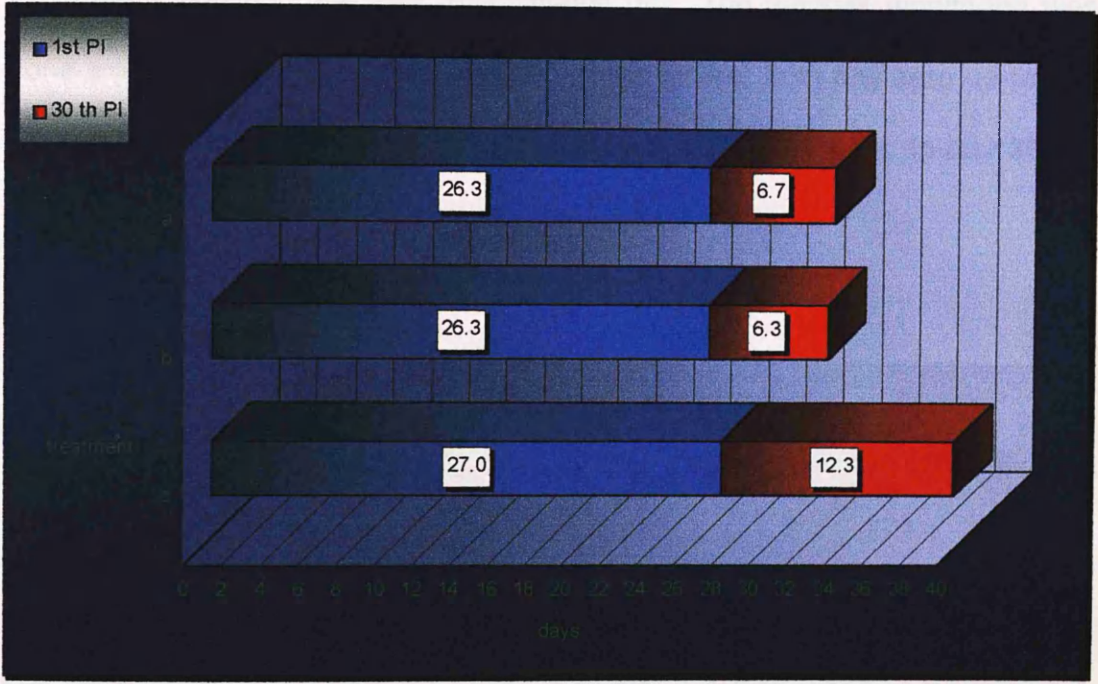


Figure 17. Average time required for the first and thirtieth metamorphosed postlarvae in each group of larvae.
Treatments a, b and c correspond to larvae hatched from embryos acclimatised at 25, 100 and 200 alkalinity levels (mg L^{-1} as $CaCO_3$), respectively. Pl = Postlarvae.

There were no significant differences ($P > 0.05$) at the time of the first postlarval appearance for the different groups of larvae. However, the time required to accomplish the 30th experimental postlarva was significantly ($P < 0.05$) longer for the treatment where the larvae came from embryos incubated at a high alkalinity concentration (200 mg L^{-1} as $CaCO_3$).

4.3.7. Larvae survival

Survival shown in Figure 18., is from the initial day of the larval experiment and until the 30th experimental day. The survival monitoring was done every 10 days of the development of the larvae, from day zero up to day 60 and even more in some cases, but it was considered only up to day 30th to have a standard day for all the different treatments.

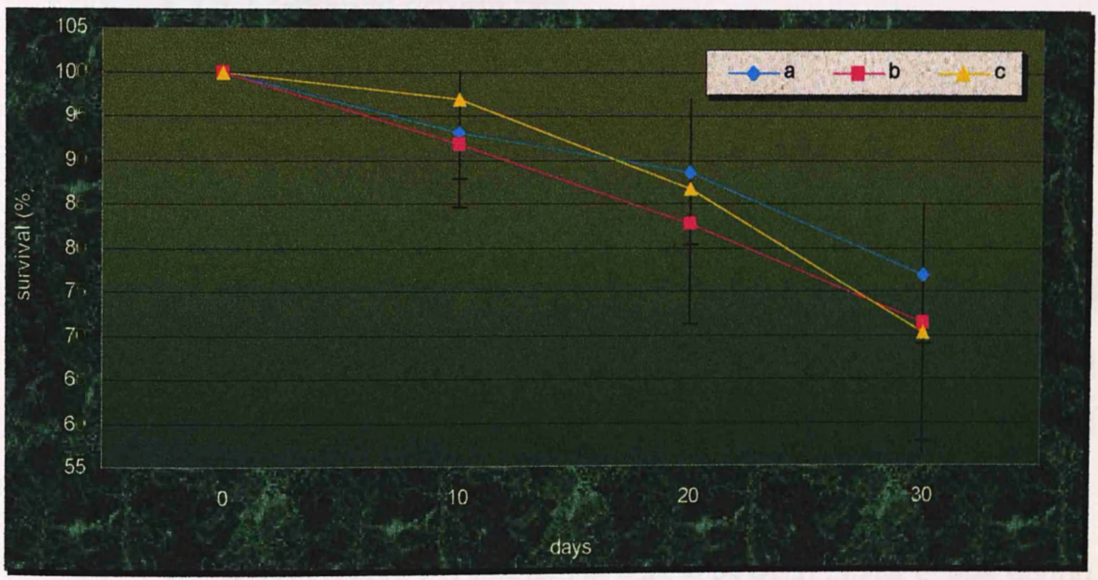


Figure 18. Percentage of survival of larvae of *M. rosenbergii* from hatching day and until day 30th of their development.
Treatments a, b and c correspond to larvae hatched from embryos incubated at alkalinity levels of 25, 100 and 200 (mg L^{-1} as $CaCO_3$) respectively.

While the assessment for survival was stopped at day 30, the collection of postlarvae for the experimental treatments (following their development after embryonic acclimatisation to different alkalinity levels) was mostly completed after this time (between days 30 and 40).

The alkalinity incubation media during the embryonic development did not have any subsequent significant influence ($P > 0.05$) in the larval development throughout the four sampling times of the larval development, which is shown in Figure 18. Similarly, the survival was not affected by the previous acclimatisation of the embryos, but, it was significantly decreased ($P < 0.05$) by the end of the experimental time, regardless of the embryonic history.

4.3.8. Postlarval growth rate

The embryonic acclimatisation of the organisms did not show any significant effect ($P > 0.05$) in their growth rate as postlarvae when they were reared in alkalinity levels of 25, 100 or 200 mg L^{-1} as $CaCO_3$. However, a trend of better growth is shown by the postlarvae produced from embryos incubated at 25 alkalinity levels in the three different treatments (Figure 19.).

The growth rate of the postlarvae was in its exponential phase during the two first months of their development. The best growth rate ($P < 0.05$) was observed from postlarvae reared in alkalinity levels of 25 mg L^{-1} as $CaCO_3$ and when they were from embryos acclimatised at 25 alkalinity levels as well.

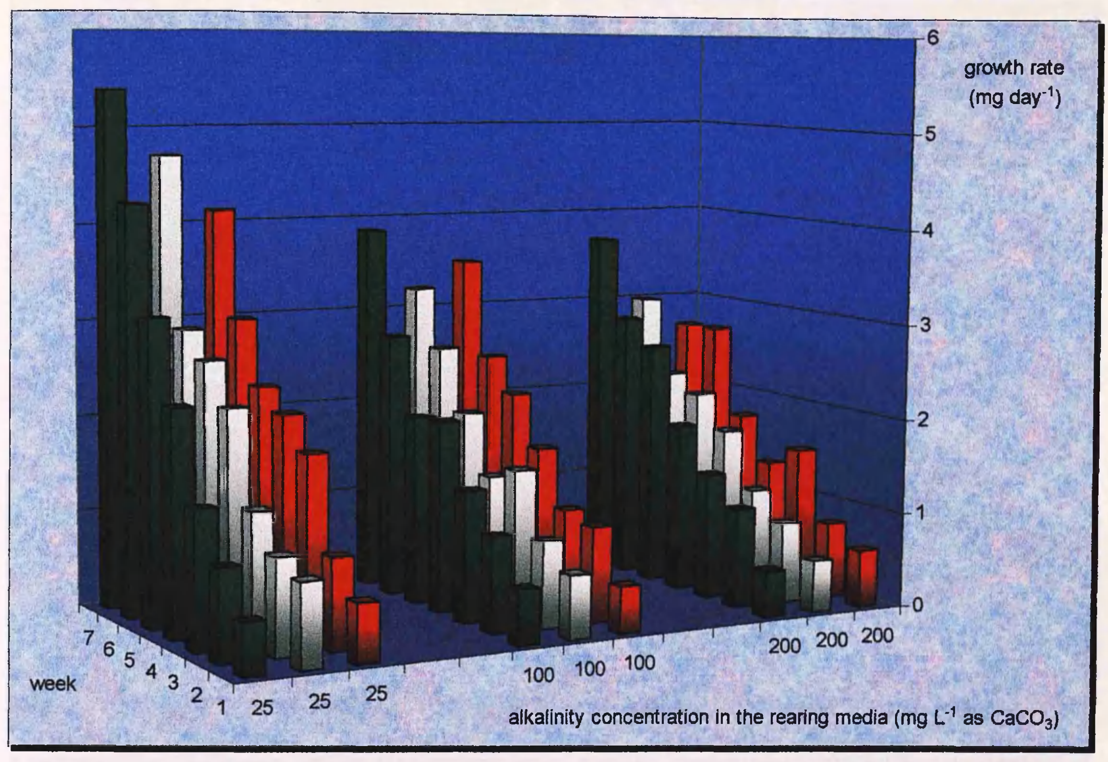


Figure 19. Growth rate of postlarvae of *M. rosenbergii* reared at different alkalinity levels with different embryonic history.
▮ postlarvae from embryos incubated at 25 alkalinity. ▯ postlarvae from embryos incubated at 100 alkalinity. ▮ postlarvae from embryos incubated at 200 alkalinity.

4.3.9. Postlarval survival

The embryonic acclimation did not have any significant effect ($P < 0.05$) on the survival of the postlarvae in any of the alkalinity levels (Figure 20.). No significant effect ($P > 0.05$) on the survival of the postlarvae was shown when they were reared at alkalinity levels of 25 and 100 mg L^{-1} as $CaCO_3$. However, the survival was significantly reduced ($P < 0.05$) when the postlarvae were reared at alkalinity levels of 200 mg L^{-1} as $CaCO_3$.

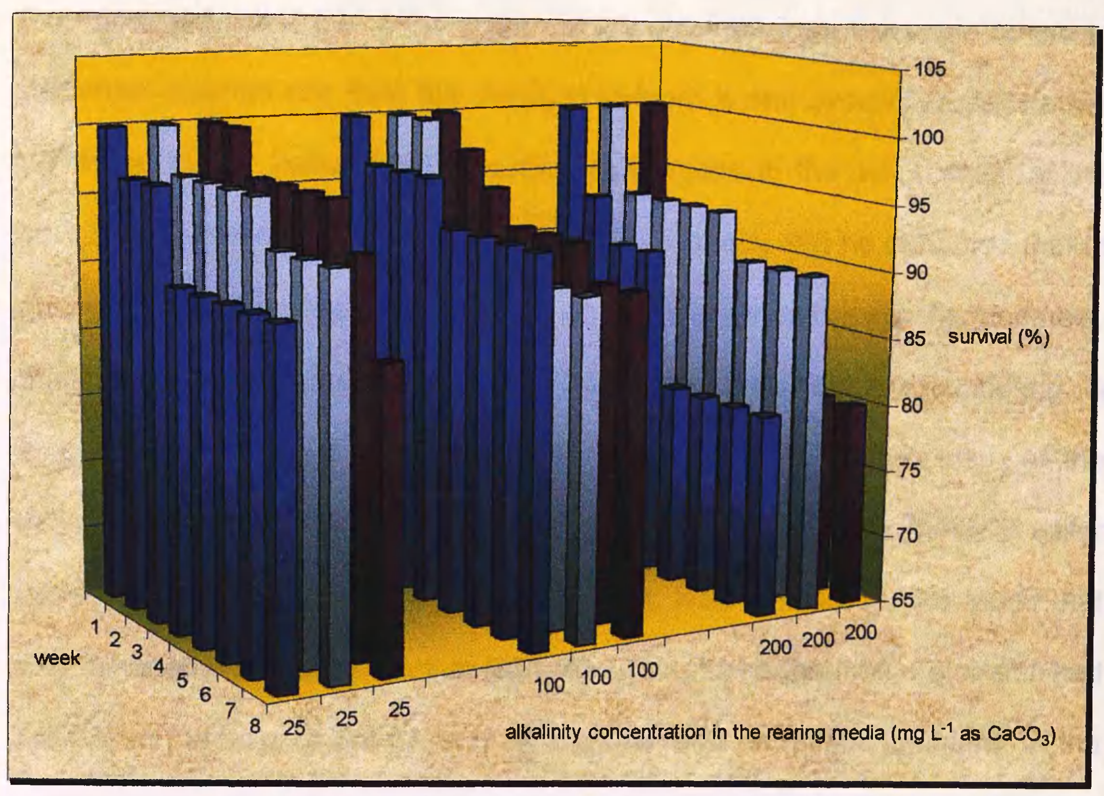


Figure 20. Survival of postlarvae of *M. rosenbergii* reared at different alkalinity levels with different embryonic history.
▮ postlarvae from embryos incubated at 25 alkalinity. ▮ postlarvae from embryos incubated at 100 alkalinity. ▮ postlarvae from embryos incubated at 200 alkalinity.

4.4. DISCUSSIONS

4.4.1. Embryonic development

According to the results of the embryonic development, there were no significant differences ($P > 0.05$) between the embryos incubated at the three different alkalinity treatments. That suggests that the embryos acclimatised and could cope with the differences in carbonates in the media. The embryos of some selachian fish require additional materials not present in

the eggs, like water and salts, and they are obtained from the environment in oviparous species and from the uterus in viviparous and ovoviviparous species (Williams, 1967). Babu (1987) reported an increase in the ash content of the crab *Xantho bidentatus* from embryonic to larval stage, and he attributed that to an increase in the copper and calcium ions during the embryonic development. Similarly *M. rosenbergii* takes calcium from the environmental water during its embryonic development (Sukadi, 1989), presumably for the formation of the exoskeleton of its first larval instar, which is accompanied by an intake of water (Balasundaram and Pandian, 1982). However, Ketola *et al.*, (1988) found that excessive environmental calcium (520 mg L^{-1}) reduced the intake of water and survival in the eggs *of Salmo salar, S. gairdneri* and *Salvelinus fontinalis.* In the present study, the embryos of *M. rosenbergii* were reared in the same calcium concentrations in the incubation media (100 mg L^{-1} as $CaCO_3$) for the three different alkalinity concentrations. The embryos were developing at the same rate and their size increment was not significantly different in the different treatments.

Ling (1969) found that the incubation period of the eggs of *M. rosenbergii* was around 19 days and the coloration started to change to slate grey after day 16th or 17th at 27 ± 1 °C. In this study, however, the duration of the incubation period was two days shorter than that as well as the change in coloration in all the three treatments. The incubation period prior to the hatching of the larvae was very similar for the organisms of the three different treatments, and that reinforces the suggestion that it could be due to the acclimatisation of the embryos to the different alkalinity levels. However, it could also be due to a

combination of the acclimatisation and the moderate calcium concentration used in the study, since Gonzal *et al.*, (1987) reported a good hatching rate of larvae of the silver carp (*Hypophthalmichthys molitrix*), at hardness levels of 400 ± 100 mg L^{-1}, whilst at hardness levels of 600 mg L^{-1} a detrimental effect was present in the hatching rate. Furthermore, Ganeswaran (1989), studied the effect of the size and age of the broodstock as well as the quality of diets and food ingestion, finding that there was no influence of any of these factors in the incubation period of embryos of *M. rosenbergii.*

Sukadi (1989) defines early stage eggs of *M. rosenbergii* to those eggs without their eyes spot and late stage eggs to those that show it. He suggests that the membrane of the early stage eggs is impermeable, whilst in the late stage eggs this membrane becomes permeable to water. He found that calcium increased from the early stage eggs to the late stage ones, when the eggs where incubated in a high hardness medium (300 mg L^{-1} as $CaCO_3$) but not at low hardness (18 mg L^{-1} as $CaCO_3$). Furthermore, he found that the size of the eggs decreased as the total hardness increased from (18 to 300 mg L^{-1} as $CaCO_3$). This, plus the results shown further ahead at the postlarval stage, suggest that the embryos used in the present study could acclimatise to high alkalinity levels, and therefore environmental carbonates as high as 200 mg L^{-1} as $CaCO_3$ had no effect in this water intake process provided the calcium of the incubation media is 100 mg L^{-1} as $CaCO_3$ or lower. However, size during the embryonic development of *M. rosenbergii* is as important as the genetic information and the nutrients passed from the broodstock to the embryo itself, since Clarke (1993) mentioned that there exists a genetic component that varies

the nutrient content in eggs of some carideans. Furthermore, Kulesh and Guiguinyak (1993) found that the first *M. nipponense* to become adults from a single clutch of eggs were those hatched from the smallest eggs. They also suggested that genetic factors are more important than any other factor when estimating development and growth heterogeneity.

In the results of this study for *M. rosenbergii*, there were no differences in the initial size and volume of the eggs, even when the females were not of an equal size and weight. Ganeswaran (1989), found that there was a positive relationship between the size of the female and the size of the eggs. However, in his study is not described if the size of the eggs was taken at the beginning of the incubation period or at any other time of that period. Similarly, Wickins and Beard (1974) found that larger females produce larger eggs. Rothbard (1977) found that there was no correlation in the number of larvae produced by a female *M. nipponese* and their weight, and they suggest that could be due to the variability in the eggs size. Manna and Raut (1991) found variations in the total weight of clutch of eggs of *M. lamarrei* and *M. dayanum*, and they suggested that the size of the egg depends on the nutritional status of the mother prawn. Therefore, the nutritional stage of the females could be the cause of the homogeneity in the initial size of the eggs used in these experiments, since all the females were fed on the same diet. Shakuntala (1977) mentioned that *M. lamarrei* develops different eggs sizes even when the broodstock is within a similar size, however he mentioned as well, that the eggs are smaller as the female gets older and the number of eggs increases when that happens. He concludes that the size of each embryo depends on the

duration of vitellogenesis, the time when the yolk is deposited, and this is when the eggs are in competition to share that yolk deposition. The females used in this study were of the same generation of prawns and of a similar age. This and the fact that they were reared in a non-stressful condition (Tropical Aquarium water systems), could have influenced the vitellogenesis period to be long enough to supply the embryos with the required nutrients and therefore give them an homogeneous initial size.

In a similar way to the size increment of the embryos, the embryonic developmental features at the four sampling days, also showed no differences between the embryos incubated from low to high alkalinity levels (25 to 200 mg L^{-1} as $CaCO_3$ respectively). Thus supporting the suggestion that the embryos could easily adapt to alkalinity levels as high as 200 mg L^{-1} as $CaCO_3$. As mentioned earlier, in the study of Ketola *et al.*, (1988) they found that eggs of salmon and trout died when incubated at calcium concentration as high as 520 mg L^{-1} as $CaCO_3$. However, they mentioned that similar eggs that were acclimatised to low calcium 34 to 64 mg L^{-1} and then transferred to high calcium waters increased their survival. That could be the same case for the embryos of *M. rosenbergii* used in the treatments with high alkalinity in this study, since they were incubated in the different water media from day one after fertilisation.

Triacylglycerides (TAG) are the preferred lipids to store energy reserves and therefore, they were measured as to find differences in energy content in the different embryos for each alkalinity treatment of this study. Lavens *et al.*, (1991) found a slight reduction in the levels of lipids during the

embryogenesis of *M. rosenbergii*, which is in agreement with the findings of Clarke *et al.*, (1990) who found that the lipid content in the embryos of the same species decreased only in a 10% from spawning to pre-hatching nauplii., Similarly, there was a slight consumption of lipids (TAG) during the embryonic development (10.056% maximum) in the present study, although no significant differences were found regarding the incubation media. Contrary to that, the polar lipids had a significant increase as the embryos were developing. Mourente and Vazquez (1996) found that early embryos and larvae of *Solea senegalensis* consumed more TAG than phospholipids, and they also found that total lipids were depleted as the embryos and larvae developed. The results shown by Badu (1987) for the embryos of *X. bidentatus* agree with the findings of this study, where the embryos of *M. rosenbergii* kept a low degree of consumption of TAG during the first two weeks of their development and then there was a high consumption during the last week of development. Badu (1987) found that 33% of the total embryonic lipids were consumed in the late stages of development of the embryos.

Sargent (1995) mentioned that TAG as well as phosphatidylcholine are consumed as an energy source during the embryogenesis and that is the case for the TAG in the eggs of *M. rosenbergii* in this study. However, the phospholipids were increasing instead of decreasing. The increase of the phospholipids, could be due to the development of new specialised cells in the embryo and therefore the necessity to create new membranes. Stryer (1988) said that the three major lipids in the composition of cell membrane are phospholipids, glycolipids, and cholesterol. Similarly to the phospholipids, the

cholesterol was increasing in the embryos of the three different treatments during the incubation period in this study. Anger and Dawirs (1981, cited in: Christiansen, 1988) mentioned that cholesterol and other related substances may act as precursors of the moulting hormone in larvae of decapod crustaceans, and they are probably the critical constituents for reserve energy saturation previous to ecdysis. Cholesterol is important in the composition of membranes, because is the moderator of the fluids that passes through them (Stryer, 1988) and therefore influencing the changes in size of the eggs.

Pigments did not show any significant difference in the development of the embryos. However, the development of the eye spots had a considerable size increment during the development of the embryo, and at the same time there was a noticeable reduction of the orange coloration (carotenoids) of the egg. Therefore, the non significant differences in the pigments content, could be due to the transference of the pigments from different parts of the egg to the eye spots (McEvoy, J., personal communications, 1999). According to Navarro *et al.,* (1992,) and Bell and Dick (1990), PC and PE are the main components of the visual tissues in some species of crustaceans. They found that 43% of PC and 32% of PE were present in the eyes of *Artemia* nauplii, making a 74% of the total phospholipids. Similarly in this study, 55 ± 5% and 30% for PC and PE (respectively) were the highest phospholipids found in the embryos of *M. rosenbergii.* Ganeswaran (1989) found that PC and PE were the most abundant phospholipids as well, and TAG the most abundant neutral lipid. That suggests that the increase of phospholipids and cholesterol levels and the maintenance of the pigments in the embryos of *M. rosenbergii* of this study was due to: a) the

preparation for the new nauplii to moult after hatching and b) the development of the eyes, which were extremely large in comparison with the rest of the body of the newly formed nauplii at the end of the embryonic development (see Plate 20."d").

Wickins *et al., (*1995) mentioned that lipid content varied in *H. gammarus* embryos proceeding from different female groups and that the rapidly developed embryos and the larvae that hatched earlier from them, contained more triacylglyceride reserves than those embryos which were slower developing and hatched as larvae in a longer period of time. The lipid content in the results of Wickins *et al.*, (1995) showed that most of the lipids were neutral lipids common to marine crustaceans, and the same was detected for this study. This could be due to the sea water-dependant metabolism of the early stages of development of the species to survive until the metamorphosis stage, where the postlarvae have to migrate to freshwater environments. Wickins *et al.*, (1995) suggested that the reduction in phospholipids in the eggs of *H. gammarus* could be due to the reduction of TAG already to a critical level when the embryos were taking longer time to hatch as larvae, and that could lead to a loss of viability. However, in this study, that was not the case for the embryos up to day 15th of their incubation period, which was very close to their hatching day. However, after the transference of the females to the brackish water (day 15th), the larvae were hatching in two batches, the first on the second day after the transfer and the second batch was taking as long as 4 days afterwards. This could probably lead to a depletion of the TAG content in those larvae that

hatched in the second batch, after transferring the embryos to the brackish water.

Parental history has a great influence in the development of the embryos and their subsequent developmental stages. Palacios *et al.*, (1998) mentioned that TAG is used to determine or infer the physiological status of the female, since this component is transferred to the egg from the ovary. However, Gomez Diaz *et al.*, (1988) said that triglycerides were not found in the muscle of *M. rosenbergii.* Lavens and Sorgeloos (1991) mentioned that High Unsaturated Fatty Acids (HUFA) levels and total lipids contents in the eggs of many species vary considerably among spawnings, even when coming from the same hatchery. They also suggested that the lack of HUFA in the broodstock can cause poor physiological development of the eggs and larvae and that the viability of the eggs depends on the quality of the food available to the broodstock. Similarly, de Caluwe, *et al.*, (1995) indicated that the levels of HUFA in the diet of the parental organism influences the development of the ofsprings. Therefore, parental history has an effect in the fuel quality and quantity in the formation of the embryo and its development, influencing the development of the larval stages, where high mortality could be present due to the lack of proper fuel energy (Palacios *et al.*, 1998). Das *et al.*, (1996) found that fecundity increases with the increase of protein content in the diets ingested by the broodstock, a diet with 40 protein and a 400 kcal / 100 g diet attained the highest fecundity in the prawns. Similarly, Murugadass and Pandian (1991) found that high protein diet produce a good development of the ovaries. In their experiment, they demonstrated that female prawns fed on 10%

protein diets succumbed before reaching sexual maturity, and females fed on diets with protein higher than 35% produced 1.6 times more eggs than those fed on less protein diets. The females used in this study were healthy and their diets were of good quality (40% protein and rich in essential fatty acids from marine origin). Therefore, it could be assumed that the period of the vitellogenesis was not disturbed by external factors, providing the embryos with the necessary nutrients and energy levels for their development. Lam (1994) mentioned that the yolk of eggs not only provides the nutrients for the development of the embryos and the newly hatched larvae, but also hormones which may serve in the regulatory functions during the ontogeny prior to endogenous hormone production. That could be the key element for the larvae to either hatch early or late, and therefore producing a fit larvae or a weak one, depending on the time spend consuming the vitelline reserves prior to its hatching.

4.4.4. Larval development

The larval development was measured according to the time taken by the larvae to reach every stage of development as well as the time required to reach their metamorphosis and the quality of the larvae at each stage of development. According to the results shown in section 4.3., it is clear that the acclimatisation of the embryos at different alkalinity levels did not affect the development of their respective successive larval stages. However, an important characteristic of the development of the larvae is that there were two

subgroups in each replicate, and that was observed after the third stage of the larval development. Guest (1979) observed that *M. amazonicum* had a homogeneous larval development until stage 6, and similarly to the observations of this study, Ling (1969) pointed out that the larval development of *M. rosenbergii* was steady up to stage 3 and then a variability of the size of the larvae was observed during his experiments. That variability could be related to the hatching time of the larvae. Shakuntala *et al.*, (1980) found that *M. lamarrei* released larvae in batches on successive nights until all the eggs held in the brood were hatched. Furthermore, they found that hatching takes place shortly after darkness and is an intermittent process, which could last until dawn. Similarly, in this study, *M. rosenbergii* preferred to hatch the larvae during the night time, although sometimes the larvae were hatching during the day time, provided the surrounding walls of the hatching tanks were darkened. That intermittent hatching of the larvae could probably give a similar condition in the differences of the larvae quality and its energetic reserves as when the larvae were hatched in different batches at different days. Anger and Spindler (1987, cited in: Christiansen, 1988) observed that larvae starved after day zero, were able to synthesise ecdysone similarly to continually fed organisms, but the lack of food was resulting in a delay in their development. Therefore, if the organisms were using more energy during the retarded hatching time, that could reduce the larval development due to the initial reduced reserves of metabolic energy.

It is possible that the less-developed larvae in all the replicates of the present study were from those embryos with less energy. Balasundaram and

Pandian (1982) found that 33% of the reserve energy of the yolk is consumed by the larvae hatched at the end of the hatching period. They mentioned that the larvae that have consumed most of the embryonic reserves are too weak, when they hatch, to predate or to escape from predators. Waddy (1977) mentioned a similar behaviour for the larvae of Homarid lobsters, where the first hatched larvae were the larger, stronger and the first to reach their postlarval stage. It could be that a similar condition is present in *M. rosenbergii* and that is why some larvae were developing more slowly regardless of the different treatments, even when they were having plenty of food (enriched *Artemia* metanauplii) supplied in the containers. Waddy (1988) mentioned that larvae of Homarid lobsters may not recover if inadequate food is supplied during the first stage of development, even if food is abundant later on. That same effect has been observed in preliminary trials for *M. rosenbergii* during this study. This could be another factor influencing the quality of the slow growing larvae encountered in all the different treatments. That is, if due to the delay of the hatching time the larvae were weak after hatching and they could not feed adequately on the enriched *Artemia* metanauplii then, they could be in the same situation as the lobsters mentioned by Waddy (1988). In which case that poor feeding during the first stage of their development could have altered their metabolism resulting in a poor condition appearance according to the score index employed. Perhaps they could reach a healthy stage of development, but taking longer time than the larvae that first hatched and were stronger to feed during the first developmental stage.

The factor of first feeding time (when the larvae start feeding on external sources of nutrients) in the development of the larvae could be more important than the source of food available to the larvae (enriched *Artemia* metanauplii), not only for the first feeding stage, but also for the whole larval development. Besides, the enriched *Artemia* spp. should be fresh to provide the required highly unsaturated fatty acids (HUFA) for the larval development. According to Evjemo and Olsen (1997) *Artemia franciscana* has more lipid (double) content than copepods but much less (half) n-3 HUFA. Besides, it is known that *Artemia* spp., lacks some of the essential fatty acids required by larvae of *M. rosenbergii*, and therefore there is a necessity to enrich the *Artemia* nauplii with a rich n-3 HUFA before is given to the prawn, which helps the development of the larvae and gives better survival (Buzzi, 1989). However, McEvoy and Sargent (1998) mentioned that enrichment has some problems in the enrichment process itself. This being autoxidation and instability, a metabolic lipid class conversion by the live-prey (*Artemia* nauplii), a retroconversion of docosahexaenoic (DHA) acid to eicosapentaenoic (EPA) acid and a loss of polyunsaturated fatty acids (PUFA) from the *Artemia* nauplii previous to their ingestion by the larvae. The autoxidation of the PUFAs can create peroxides, which are potentially toxic oxidation products for the larvae if the enrichment is not ingested in less than 24 hours, preferably 18 to 19 hours after its application to the *Artemia* nauplii (McEvoy *et al.,* 1995). The aeration in the culture tank is the principal catalytic agent for the autoxidation of the enrichment. McEvoy and Sargent (1998) mentioned that a disenrichment occurs if the *Artemia* nauplii are starved for a period of 24 hours and they suggest to keep the enriched *Artemia* spp. in a low temperature, to reduce the conversion

of DHA into EPA, as well as to prepare newly enriched nauplii every 12 hours. During the present study the maximum time delay in the enriched *Artemia* spp. production was 24 hours, and as suggested by McEvoy and Sargent (1998) the *Artemia* metanauplii were kept in a refrigerator for the late feeding routine.

It is not clear why this differences in the hatching times of larvae from one batch of embryos occur, however, parental history and the embryogenesis period have a definite key role in the development of the last embryonic development prior to the hatching of the larvae. Balasundaram (1993) found that larvae of *M. rudi, M. malcolmsonii* and *M. scabriculum* hatched their larvae in different batches in a period of 4 ± 2 days. In the experiments of Wickins and Beard (1974) a total of 18 females hatched the larvae within one batch and 13 within 2 batches. The latter authors did not find differences in the size and development of the larvae hatched in the first or the second group from the same female. Similarly Wickins *et al.*, (1995) did not find differences in the development or survival from early or late hatched larvae of *H. gammarus.* That seems to be contradictory, since some authors mention (Waddy 1977; Balasundaram and Pandian, 1982), that the second group of larvae have less energy reserves and therefore a lower rate of larval development by the reasons explained above. New (1995) mentioned that changes in the larval development and the differences in their growth are mainly due to genetical influence instead of environmental factors, which is linked to the embyogenesis. Gomez Diaz and Nakagawa (1990) mentioned that the diet of *M. rosenbergii* influence the fatty acids content in the organisms itself, and that, as mentioned earlier, can influence the content of fatty acids included in the eggs and the

subsequent development of the embryo and the larval stages. Thus resulting in differences of the performance of the organisms and that can be a cause for the different subgroups of larvae present in the same batch. Therefore, apparently the health history of both progenitors, genetic information transmission and the nutrients provided by the female during embryogenesis, affect more directly the performance of the larvae than any other external factor influencing their development after hatching. Thus including the quality of the first food available, since Cook and De Baissac (1993) found that no significant differences were found in larvae of *M. rosenbergii* reared with phytoplankton or *Artemia* spp. diets and de Caluwe *et al.,* (1995) mentioned that larvae of *Macrobrachium* spp. obtained from females fed a HUFA enriched diets had a similar good development when fed with enriched *Artemia* spp. as when fed with non enriched *Artemia* spp.

In this study, the assessment of the larval development was adjusted due to the subjectivity of the model used. Aquacop (1983) mentioned that it was easy to control a specific bacteria, that could cause disease in an open system, by drying the system just before the next cycle of production and by adding antibiotics to the systems just before the metamorphosis. They mentioned that care should be taken in 8 different points during the larval development: gut content, missing appendages, necrosis, external bacteria, food chasing, swimming activity and general colour, the last three characteristics could be seen with the naked eye. This makes it easy to standardise when working with lots of larvae in a short period of time, like in commercial hatcheries. However, they report that is not necessary to worry in a closed system where the

potentially harmful bacteria could be controlled by a constant population of non harmful bacteria in the system, and where is no necessity of antibiotics, giving with this high survival and metamorphosis rates. Similarly in this study the larval condition index was modified as mentioned in section 4.2.9.7.1.1., due to the subjectivity of some parameters, such as the gut fullness (depending on the time of sampling), the lipid content (state of the hepatopancreas), the abdominal muscle appearance (subject to light intensity), the swimming behaviour (depending on stage of development) and finally as mentioned by Aquacop, none of the larvae sampled had any fouling organisms.

It was found that up to five larval stages were existing in a particular LRC when the first postlarvae were observed and in those groups the main developmental larval stage was 9, and by simple eye selection the score could be biased. In caridean prawns, external factors e.g. temperature, have been related to variations in the number of larval moults (Knowlton 1976; Rochanaburanon and Williamson 1976, both cited in: Gomez Diaz and Kasahara, 1987) and according to Williamson (1982) the minimal number of moults should occur under optimal conditions for survival. Excessive moults commonly produce repeated developmental stages without size increment (Gomez Diaz and Kasahara, 1987). However, the external conditions were the same for all the larvae reared in each LRC, so the reason for the accumulation for several instars should be endogenous and mostly related to the parents history as previously discussed. An interesting observation was that the larvae were staying in stage 5 of their development and again in stage 8 for several days, resulting in a temporary homogeneity of the larval development. There is

not a clear explanation for that phenomena. However, Read (1984) mentioned that the larvae of *M. petersi* osmoregulate better during the first larval and postlarval stages of their development, and that stages 2, 5 and 9 showed remarkable powers for osmoregulation as well. Therefore, perhaps the fifth larval developmental stage of *M. rosenbergii* was delayed from any further development due to the metabolic changes for the osmoregulation of the animal, and being the same for stage 8. According to Harntoll and Dalley (1981) the larvae of *P. elegans* have high variability in the five first instars, and then in the sixth larval and first postlarval instars, the variation diminished considerably. It could be then, that a similar response of *M. rosenbergii* is a strategy to reduce the high variability of the size increment of the organisms at those intervals to ensure a homogeneous size in the groups of larvae to reduce cannibalism and feeding or space competition. Whilst at the same time preparing themselves for the metamorphosis process and its metabolic changes for the osmoregulation when migrating to freshwater environments. Surely there is a need of more research to find out the physiological changes of the organisms at those two intervals during their larval development.

Although there were no differences in the development of the embryonic stage when incubated at different alkalinity levels, the rate of metamorphosis was different for the larvae hatched from embryos incubated at an alkalinity level of 200 mg L^{-1} (as $CaCO_3$). Kulesh and Guiguinyak (1993) demonstrated that there are differences in the development of the larvae of *M. nipponense*, from a single clutch of eggs, where the first berried females were from the larvae that first metamorphosed into postlarvae. Gomez Diaz and

Ohno (1986), found that larvae hatched in the second batch of a single clutch of eggs and reared under optimal temperature conditions (28 °C.), had a higher percentage (93.8%) of metamorphosis than those hatched during the first batch. This suggests that although the larvae from the first hatched batch are stronger and with more energy reserves than the successive batches, it could be a genetically transferred quality that induces a better development of the larvae. In this study, the larval developmental stages were not different from larvae hatched from embryos incubated at different alkalinity levels. Besides, there were no differences in the biochemical composition of all the embryos. However, it seems that the rate of metamorphosis was affected by the incubation conditions of the embryos previous to the larval development, since the larvae from embryos incubated at higher alkalinity levels did require a longer period to reach metamorphosis.

Larval experiments for *M. rosenbergii* could give different survival rates even when the organisms are treated in identical treatments (Aniello and Singh, 1982). However, in this study, no significant differences in the survival of the larvae of *M. rosenbergii* were found for any of the different alkalinity treatments. Similarly, Romdhane *et al.*, (1995) found that there were no significant differences in the survival of larvae fed with enriched *Artemia* nauplii and unenriched *Artemia* spp. Therefore, that suggests that the survival could be affected not only by the studied variable itself, but by intrinsic factors such as genetic characteristics of the larvae and parental history as well as by the initial strength of the larvae after hatching and the quality of the first feed. Fujimura and Okamoto (1970) found that daily mortality increased after day 25th of the

larval development and they suggested that it was probably due to decomposition of the uneaten food causing pollution of the water. Furthermore, they mentioned that the larvae at stage XI are susceptible to direct sunlight and therefore they can not eat and then high mortality occurs. However in this study, there was no direct sunlight affecting the larval development and survival, furthermore, the cleaning and feeding of the organisms was not a problem to cause pollution of the rearing system.

4.4.7. Postlarvae development

Although there were no significant differences in the development of the postlarvae due to the alkalinity in the rearing media, a marked trend was shown as mentioned in section 4.3.8., where the postlarvae metamorphosed from larvae hatched from embryos incubated at alkalinity of 25 (mg L^{-1} as $CaCO_3$) were the ones with the highest growth rate, regardless of the rearing alkalinity concentration. The postlarvae with lower growth rate however, were the ones from embryos acclimatised at an alkalinity concentration of 200 mg L^{-1} as $CaCO_3$. The lack of significant differences in the postlarval development of the organisms was due most probably to the variability in the growth rate of the individual postlarvae within the replicates. Ra'anan and Cohen (1984b) mentioned that larvae may metamorphose into postlavae in a range of 3 to 7 days, but the "jumpers" could be metamorphosed postlarvae, which were not the largest and neither the first metamorphosed. However they did not explain the reason for the appearance of the "jumpers". Brown *et al.*, (1991) mentioned

that social behaviour influenced the growth of the postlarvae in a community and that could have happened in the groups of postlarvae in the meshpots of this study, where high variability in size increment was found in each sub-population (replicates) every sampling week.

Gomez Diaz (1987) mentioned that the results of acclimatisation of embryos of *M. rosenbergii* could increase the tolerance limits to temperature and salinity of the larval and postlarval stages of *M. rosenbergii*. However in that case he mentioned that the alteration of the tolerance limits could have been influenced by various factors like the historical development of the parents, i.e. environmental conditions previous to the maturation of the gonads, genetic differences among each parent an differences in their diets as well. He used only one female per acclimatisation treatment, so his results could have been affected by the individual physiological state of the parental organisms, e.g. it could be that weak embryos of an unfit parent acclimatised to 29 °C. would not tolerate wider range of temperature than those produced by a physiologically good broodstock and acclimatised to 25 or 31 degrees, as he found. In the present study, the external characteristics of the broodstock and the records maintained for them were showing a healthy standard for each female and male used as breeding organisms, besides there were three replicates for each batch of embryos produced for each treatment. However, any physiological or genetic problems were unknown, and that could influence the variability of the ofspring growth rates. The results of section 4.3.8 showed a clear trend in reduction of the growth rate of postlarvae metamorphosed from larvae hatched from embryos incubated at higher environmental alkalinity, however, no significant

differences were found. In the other hand, the postlarvae reared in the higher environmental alkalinity showed a significant reduction in the growth rate, regardless of their embryonic incubation media. Thus indicates that although the embryos were capable of acclimatise and tolerate high alkalinity levels, the effect of high alkalinity levels was lethal for most of the postlarvae.

The survival of the postlarvae was negatively affected in the treatment with the higher alkalinity concentration (200 mg L^{-1} as $CaCO_3$). In this section of the study, the environmental factors and management were controlled in a similar way as to the first experiments, and most of them are unlikely to be the reason for the higher mortality in high alkalinity levels, except alkalinity itself. Merchie *et al.*, (1995) suggested that under suboptimal conditions, vitamin "C" enriched diets could enhance the stress resistance of larvae and postlarvae of *M. rosenbergii.* Therefore, it could be that the postlarvae fed on green beans (rich in vitamin "C") could be well nourished and therefore resistant to the effects of the high alkalinity levels in the water. However, the results from the first experiment showed that even when the postlarvae of that experiment were fed with the same diet they had a lower survival rate, so even with the reinforcement of vitamin "C" the postlarvae were unable to deal with the effect of high alkalinity. The ultimate purpose of this section of the study was to investigate if an increase in the alkalinity of the embryonic incubation media could lead to postlarvae capable of tolerate higher alkalinity levels, and the results showed that that was not the case.

4.5. CONCLUSION

Alkalinity did not have any statistical significant effect in the development of the embryos of *M. rosenbergii*. No differences were found in the weekly development of the embryo and neither in their triacylglyceride content as energy expenditure.

The larval growth rate, their stage of development and survival were not affected by the embryonic acclimatisation to the environmental alkalinity. However, the metamorphosis period was longer for larvae hatched from embryos acclimatised to high environmental alkalinity.

The postlarvae of *M. rosenbergii* with the best growth rate were the ones that were born after embryos incubated at low levels of alkalinity, regardless of their rearing alkalinity. However, prior exposure to increased alkalinity as embryos had no effect on the ability to resist high alkalinity subsequently.

CHAPTER 5

5. SUMMARY

Growth rate, survival and length-specific dry weight were reduced by the increase of the environmental alkalinity, whilst moult frequency and magnesium content were increased as the environmental alkalinity increased. Moult frequency and magnesium content were also increased as the environmental total hardness increased.

In response to the results of the first part of the study, it is suggested that the optimum level of alkalinity is around 70 mg L^{-1} as $CaCO_3$, (between 50 and 100). Total hardness is not a problem for the development of the prawns as long as the simultaneous alkalinity levels are lower than 100. So provided the alkalinity is lower than 150 and preferably lower than 100, the postlarvae can grow and survive even if the total hardness is as high as 1000 mg L^{-1} as $CaCO_3$. Therefore, alkalinity (as $CaCO_3$) should be one of the criteria considered for the selection of *M. rosenbergii* production sites. Sites with high environmental hardness should not be necessarily avoided for the culture of the species if the alkalinity concentration is low.

Since alkalinity had a greater effect than the total hardness in the development of the postlarvae of the species, the second part of the study focused in a possible adaptation of the species to different levels of alkalinity from its early embryonic stages of development, to find out if the postlarvae could increase their tolerance limits to that environmental factor.

The embryos of *M. rosenbergii* did not show any difference in their development or energy consumption (TAG), and were able to cope with environmental alkalinity levels as high as 200 mg L^{-1} as $CaCO_3$.

The larval development of the species did not show any significant response to embryonic acclimatisation to different environmental alkalinities, however, larvae hatched from embryos incubated at high alkalinity levels had the slowest rate of metamorphosis.

The acclimatisation of the embryos did not influenced the growth rate of the postlarvae and neither their survival at different environmental alkalinity concentrations.

Although the transference of the newly postlarvae was gradual and from metamorphosis, the embryonic acclimatisation to high environmental alkalinity did not prevent the lethal effect of high alkalinity levels in the rearing of the postlarvae.

The results of the second part of the study confirm the results of the first one, where it was concluded that high environmental alkalinity is a limiting factor for the culture of the species. Therefore, other alternatives must be chosen in order to improve the use of water with high levels of alkalinity, specially above 200 mg L^{-1} as $CaCO_3$.

It was noticed that some postlarvae were dying after the third experimental moult and as discussed earlier, that time was related to their 8th to 9th week after metamorphosis. Sukadi (1989) suggested that these organisms suffer a physiological change in their system around that time, and according to the results of the experiments of the first part of this thesis, it could be that physiologically weak organisms died without being able to cope with the effects of alkalinity and total hardness due to this internal changes. Therefore, it is recommended to investigate the existence of such a physiological change in the organisms, since is a time when most of the postlarvae are regularly transferred from hatcheries and nurseries to the grow-out ponds.

An interesting observation on the development of the larval stages of *M. rosenbergii* was made in this study. It was noted that during two periods of time the larvae stay steady in one stage of development, and it has been suggested that is due to an own species strategy to homogenise the population and avoid future cannibalism and competition. However, more research is needed into it, to find out why the larvae really stay for a longer period of time in stage five of their development and then again around stages 8 and 9 prior to metamorphosis. Since this has implications for hatcheries in the point of view that less variability in the larval development could produce bigger amounts of postlarvae at a single day after metamorphosis and therefore a better management control before stocking or selling them.

The results of the second part of this study showed that there was a trend of better growth rate in the response of the postlarvae from embryos

acclimatised to low alkalinity concentrations. This suggests that a longer experiment (using at least one generation) would be advisable to find out if the acclimatisation of the broodstock into higher alkalinity could influence the vitellogenesis and with it, the transference of any factor helping in the acclimatisation of the progeny to different alkalinity levels.

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APPENDICES

Appendix I,a

CONSTRAINTS ENCOUNTERED IN THE TRIALS PREVIOUS TO THE EXPERIMENTS OF CHAPTER 3

Several attempts to run the experiments of chapter 3 were conducted under a different experimental system (a semi-static water flow or partial water exchange system), which gave a series of inconveniences for the development of the study.

The experimental system

This system was followed the design of a previous experimental system used in Gonzalez Vera (1995) and a brief description is given here. The partial water exchange system consisted of transparent plastic tanks (aquariums) of 39 L* x 20 W* x 25 H*. The tanks were placed on heater plates, which could hold a maximum of 4 tanks at the time, and 9 different treatments containing 3 replicates each were run at the same time. Each tank was filled with 8 L with the chemically prepared water deionized media (see section 2.6. for reference to the chemicals used to prepare the water media). The water media was changed every 48 hours and the food leftovers were collected early in the morning and late in the evening. The organisms were held in meshpots as described in section 3.2.2.2. Each tank was individually air supplied through

an airstone and a communal air tube. The use of that system during the first trials of the alkalinity and total hardness experiments had the following constrains:

Physico-chemical constraints

a) One of the most important constraints was the continuous stress to the experimental organisms, due to the water exchange every 48 hours. Although the water exchange was done in the fastest possible way and as careful as possible, the organisms were keep out of the water in every water exchange.

b) Water exchange was extremely exhausting and time consuming, since 27 trays containing 3 prawns in each one had to be changed every 48 hours, and newly cleaned and empty trays prepared before transferring the organisms.

c) Every tray had to be filled with the exact amounts of deionized water and chemicals to make up the total hardness and alkalinity required before the organisms were transferred, giving a considerable human error in the final results.

d) The temperature of the newly prepared water media had to be maintained at the same temperature of each previous tank where the

postlarvae were hold, which was inaccurate and again extremely high time consuming.

e) Variations in the air temperature had a great influence on the variability of the temperature of the different replicates for each treatment. This was unavoidable, since the experimental room was opened constantly for several reasons (transporting of daily feeding rations, measuring instruments, etc.) and the difference between the outside air temperature and room temperature was between 5 and 10 °C, depending on the time of the year.

f) The fact that each treatment had one or two different heating plates was another factor influencing the variability of the experimental temperature among the different treatments.

g) Evaporative water loses, unarguably influenced changes in the chemical concentration of the different elements in the water column for each replicate and treatment, being gradually reduced from the day the water was newly replaced to the 48 hours after it was changed.

h) Although the uneaten food was carefully removed every morning and evening to reduce its pollution effect in the water column, accumulation of the metabolites produced by the postlarvae pulluted the water media in a fast way, increasing rapidly the levels of total ammonia.

Biological constraints

a) An infestation of fungal filamentous *Saprolegnia* species (Austin and Austin, 1989) was appearing in the uneaten food in several trays, when the food was left overnight. The only two possible carriers of the fungal spores were:

1) The unfiltered water used in the deionizer, previous to the preparation of the experimental water media, and

2) The dry pellets used at the beginning of the preliminary trials, which were stored for sometime in the Tropical Prawn Unit from previous experiments.

b) A third trial was started after high mortality in the two previous ones, but unfortunately it had to be stopped as well, since bacteria was infesting the organisms this time. This infection was present in a particular area located on the outer side of the carapace, just above the heart position (Figure I,a.). The appearance of the spot was circular and light blue coloration, of approximately one third of the

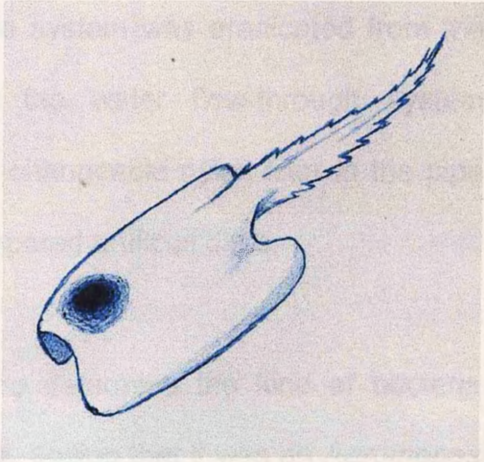

Figure I,a. Carapace of *M. rosenbergii* infected by bacteria possibly *Aeromonas hydrophila.*

width of that portion of the carapace. The spot increased in size within 2 to 3 days to cover almost the whole wide of the carapace in the same particular area, gaining a darker brown coloration, and by the sixth to eighth day, just before the moulting, the pathogen organism had already eroded the carapace. Some organisms were able to survive and get rid of the parasitic bacteria along with the shedding of the old exoskeleton and some of them were able to reach relatively good growth rates of up to 4 mg day^{-1}. Unfortunately, occasionally some postlarvae were infected on the tip of the gills as well, showing a similar brownish coloration on them. Majority of these gill infected organisms were dying during their next ecdysis, but again, some of them could shed the exoskeleton along with the bacterial infection. This biological phenomenon was present in all the different treatments, regardless of the variations in the calcium or carbonate content of the water column.

All the physicochemical constraints were reduced or eradicated by the implementation of a water flow-through system, which is described in section 3.2.2.1. The fungal infestation of the system was eradicated from the experiments after the implementation of the water flow-through system mentioned earlier, the addition of a 1 μm re-changeable nylon filter in the pipe of the water supply and the use of freshly prepared artificial diets.

Bacteriological tests were used to determine the kind of bacteria present in the system affecting the postlarvae, finding that it was an *Aeromonas* spp., with high possibility of being the species *A. hydrophila / caviae or A. sobria*

(BioMérieux computerised test) (Del Rio Rodriguez, R. personal communications, 1997). However, it could be another identified species, *Vibrio fluvialis.* El-Gamal *et al.*, (1986) found a similar bacteriological disease for *M. rosenbergii*, as the one described in this study. They identified an exoskeletal lesion, in the form of a black burn spot, associated with the same chitinoclastic bacteria, *A. hydrophila.* Furthermore, they mentioned that in order to get rid of an infected area in the appendages, the prawns could commit autotomy, this being similar to the shedding of the exoskeleton by the postlarvae of this study. Some other genera of bacteria have been reported to infest *Macrobrachium* species. Lacroix *et al.*, (1994) found that *Rikettsia* spp. was the cause of the high mortality in *M. rosenbergii* culture. Bacterial diseases can cause severe damage to the exoskeleton and then to the muscle of the prawns as well as to the gills. Lombardi and Labao (1991a,b) reported that chitinolytic bacteria, *Pseudomonas* spp., *Vibrio* spp., *Beneckea* spp., *Aeromonas* spp. *and Leucothrix* spp. are common in hatcheries causing black spot bacterial necrosis and gill obstruction. Natarajan *et al.*, (1982) mentioned that wild caught Indian *M. equidens* were infected by chitinoclastic bacteria such as *Pseudomonas* spp., *Bacillus* spp., *Aeromonas* spp., and *Serratia* spp. Brady and Lasso de la Vega (1992) isolated bacteria, predominantly *Aeromonas* spp., *Bacillus* spp., and *Pseudomonas* spp., from physically damaged freshwater prawns *M. rosenbergii*, whilst no bacteria were found in prawns without lesions. The genus *Alcaligenes* spp. and *Vibrio* spp. have been identified in the tissues of larval *M. rosenbergii* in some Malaysian hatcheries (Anderson *et al.*, 1989).

In 1989, Austin and Austin described a series of diseases caused by micro-organisms in crustacean. A described shell disease of decapod crustaceans (shrimps, lobsters, crabs and some other species) named as burn spot, brown spot or rust spot disease, appears as an erosion of the cuticle, gills or appendages. They mentioned that it could be caused by a variety of bacteria, which produce different extracellular enzymes like proteases, lipases and chitinases. *Aeromonas* spp., *Flavobacterium* spp., *Spirillum* spp., and *Vibrio* spp., are believed to be the causative pathogens for the brown spot disease in *Penaeus* spp. shrimps (Lightner, 1988, cited in: Austin and Austin, 1989). Yasuda and Kitao (1980) found that *Vibrio* spp. was the dominant natural intestinal flora for zoea stages of *P. japonicus,* but *Pseudomonas* spp. were the dominant ones when the shrimps were in the adult stage. However, they found high infestation with *Aeromonas* spp. and *Vibrio* spp. in the digestive tracts of poorly growing shrimps. Baticados *et al.*, (1986) experimented with *P. monodon* to induce soft-shelling organisms, infesting them with chitinoclastic bacteria, but the results were negative, although some soft-shell caught organisms were infected with *Vibrio* spp. and *Aeromonas* spp.

As mentioned earlier, burn spot disease is a common problem in crustacea culture and mainly in intensive cultures. However, the interesting characteristic of the disease encountered in this study is that in this case it was present specifically on the previously described carapace area and occasionally on the tip of the rear gills.

The dead organisms observed in this study were mostly in their ecdysal stage, and that could be related to the environmental condition in conjunction with the bacterial disease. Stern and Cohen (1982) mentioned that some changes in the oxygen consumption and ammonia excretion occur during the moult cycle. They found that oxygen consumption rises from 1.3 to 3 mL O_2 g^{-1} per hour, and ammonia excretion increases from 17.9 to 39.3 g NH_3-N g^{-1} per hour from intermoult to ecdysis stages in juvenile organisms of *M. rosenbergii.* This indicates that at ecdysis the animals are in the poorest condition, since depletion of oxygen in the system causes an increase in the toxicity of ammonia (Wajsbrot *et al.*, 1990), and that increases the stress suffered by the organisms during the moulting process itself. Furthermore, if some species of bacteria were present in the tissue of the experimental larvae of *M. rosenbergii* (Anderson *et al.*, 1989), it is then probably that an outbreak of the bacteria in the postlarval stage occurred once the organisms were highly stressed by the poor environmental conditions of the water previous to the water exchange, the stress caused by the moulting process and handling during the water exchange, the nature of the chemical composition of the water column and the possible contamination of the old artificial pellets. In agreement with that, El-Gamal *et al.*, (1986) mentioned that most of the reasons attributed to this kind of bacterial infection in cultured prawns are handling, poor water quality, high stocking density and dietary deficiency. However, Harpaz and Schmalbach (1986) reported a considerable reduction of black spot disease incidence in *M. rosenbergii* when its artificial diet was supplemented with fresh green leaves, which in this study were supplied as green beans.

According to the findings of the authors mentioned above, it seems that the disease found in this study was a variation of the burn, brown, black or rust spot disease in crustacean, since it is caused by a chitinoclastic bacteria and the symptoms are very similar to all those mentioned. The bacterial infestation was not deeply studied here, since it was not longer present in the subsequent experimental postlarvae populations used in this study. However, in terms of economical risks for hatcheries of *M. rosenbergii*, further research is necessary in order to identify the causative factor as well as the path of the invasion of such bacteria in some postlarvae populations of this species. Furthermore, it is necessary to find out why it was limited to that particular area of the organisms and how some organisms managed to get rid of the disease during the ecdysis stage.

Appendix II,a

Water quality from tap water supplied by East of Scotland Water; water supply zone ZN02, 1-Jan-97:31-Dec-97.

Parameter	Units	N. of samples taken	Concentration		
			Min.	Mean	Max.
Colour	mg/LPt/Co	8	<0.5	1.45	2.8
Turbidity	Ftu	8	0.15	0.25	0.35
Colour (quantitative)	DIL N.	8	1	1	1
Taste (quantitative)	DIL N.	8	1	1	1
Temperature	°C	14	0	9.84	17.3
Hydrogen ion pH	pH value	20	7.3	7.84	8.89
Sulphate	mg L^{-1}	9	10.10	11.43	12.85
Magnesium	mg L^{-1}	1	1.13	1.13	1.13
Sodium	mg L^{-1}	1	2.12	2.12	2.12
Potassium	mg L^{-1}	1	0.19	0.19	0.19
Dry residues	mg L^{-1}	1	49	49	49
Nitrate	mg L^{-1}	9	0.46	0.87	3.05
Nitrite	mg L^{-1}	8	<0.003	<0.003	<0.003
Ammonium	mg L^{-1}	8	<0.005	0.01	0.011
Oxidizability (permanganate value)	mg L^{-1}	1	0.71	0.71	0.71
Total organic carbon	mg L^{-1}	1	1.15	1.15	1.15
Total Phenols	µg L^{-1}	4	<0.07	<0.07	<0.07
Aluminium	µg L^{-1}	8	11	48.12	87
Iron	µg L^{-1}	8	5	42.5	193
Manganese	µg L^{-1}	8	<1.5	6.51	14.5
Copper	µg L^{-1}	1	<0.1	<0.1	<0.1
Zinc	µg L^{-1}	1	<0.1	<0.1	<0.1
Phosphorus	µg L^{-1}	8	<8	17.87	85
Fluoride	µg L^{-1}	4	<8	9.5	14
Silver	µg L^{-1}	1	<1	<1	<1
Colour qualitative (Nature)	-	12	–	-	-
Taste qualitative (Nature)	-	12	–	-	-
Arsenic	µg L^{-1}	1	<1	<1	<1
Cadmium	µg L^{-1}	1	<0.4	<0.4	<0.4
Chromium	µg L^{-1}	1	<0.8	<0.8	<0.8
Mercury	µg L^{-1}	1	<0.05	<0.05	<0.05
Nickel	µg L^{-1}	1	<1.5	<1.5	<1.5
Lead	µg L^{-1}	4	<1.5	2.45	5.3
Antimony	µg L^{-1}	1	<5	<5	<5
Selenium	µg L^{-1}	1	<1	<1	<1
Polycyclic aromatic hydrocarbons	ng L^{-1}	4	<4	7.81	14.69
Fluoranthene	ng L^{-1}	4	<4	6.67	14.69
Total Coliforms	No./100 mL	28	0	0	0
Faecal Coliforms	No./100 mL	28	0	0	0
Faecal Streptococci	No./100 mL	4	0	0	0
Colony counts at 22 °C	No./1 mL	28	0	2	48
Colony counts at 37 °C	No./1 mL	28	0	0	0
Residual disinfectant - Free	mg Cl_2 L^{-1}	28	-	-	-
Residual disinfectant - Total	mg Cl_2 L^{-1}	28	-	-	-
Conductivity	µs cm^{-1}	12	58	62.83	67
Chloride	mg L^{-1}	9	3.3	4.54	5.7
Calcium	mg L^{-1}	1	7	7	7
Boron	µg L^{-1}	1	<50	<50	<50
Barium	µg L^{-1}	1	8	8	8
Benzo 3,4 pyrene	ng L^{-1}	4	<1	<1	<1
Tetrachloromethane	µg L^{-1}	4	<1.6	<1.6	<1.6

Appendix II,a (Continuation)

Parameter	Units	N. of samples taken	Concentration		
			Min.	Mean	Max.
Trichloroethene	µg L^{-1}	4	<2	<2	<2
Tetrachloroethene	µg L^{-1}	4	<0.3	<0.3	<0.3
Totla trihalomethanes	µg L^{-1}	4	14	23.53	32.41
Trichloromethane	µg L^{-1}	4	12.2	20.77	28.5
Dichlorobromomethane	µg L^{-1}	4	1.82	2.76	3.87
Dibromochloromethane	µg L^{-1}	4	<2	<2	<2
Tribromomethane	µg L^{-1}	4	<1.5	<1.5	<1.5
Total Hardness	mg Ca L^{-1}	1	11	11	11
Alkalinity	mg HCO_3 L^{-1}	1	14	14	14
Aldrin	µg L^{-1}	1	<0.01	<0.01	<0.01
Alpha-HOH	µg L^{-1}	1	<0.01	<0.01	<0.01
Atrazine	µg L^{-1}	1	<0.01	<0.01	<0.01
Carbophenotion	µg L^{-1}	2	<0.01	<0.01	<0.01
Dieldrin	µg L^{-1}	1	<0.01	<0.01	<0.01
Endrin	µg L^{-1}	1	<0.01	<0.01	<0.01
Fenitrathion	µg L^{-1}	2	<0.01	<0.01	<0.01
Gamma-HOH	µg L^{-1}	1	<0.01	<0.01	<0.01
Heptachlor	µg L^{-1}	1	<0.01	<0.01	<0.01
Hexachlorobenzene	µg L^{-1}	1	<0.01	<0.01	<0.01
Malathion	µg L^{-1}	2	<0.01	<0.01	<0.01
Parathion	µg L^{-1}	2	<0.01	<0.01	<0.01
Ppentachlorophenol	µg L^{-1}	4	<0.07	<0.07	<0.07
Propazine	µg L^{-1}	1	<0.01	<0.01	<0.01
Simazine	µg L^{-1}	1	<0.01	<0.01	<0.01
Trifluralin	µg L^{-1}	1	<0.01	<0.01	<0.01
Endosulphan A	µg L^{-1}	1	<0.01	<0.01	<0.01
Endosulphan B	µg L^{-1}	1	<0.01	<0.01	<0.01
Fenthion	µg L^{-1}	2		0	
op'-DDE	µg L^{-1}	1	<0.01	<0.01	<0.01
op'-DDT	µg L^{-1}	1	<0.01	<0.01	<0.01
pp'-DDE	µg L^{-1}	1	<0.01	<0.01	<0.01
pp'-DDT	µg L^{-1}	1	<0.01	<0.01	<0.01
Trietazine	µg L^{-1}	1	<0.01	<0.01	<0.01
Phenol	µg L^{-1}	4	<0.05	<0.05	<0.05

Appendix II,b

Formulation of enrichment emulsion for *Artemia* nauplii, modified from Watanabe *et al.*, (1982).

Fish oil	60 %
Water	28 %
Tween 80	10 %
Soya Lecithin	2 %
Vitamin E	1.5 % of the oil weight
Cholesterol	2 % of the oil weight
Astaxanthin	0.17 % of the oil weight

Astaxanthin was diluted in 10 to 15 mL of the total water volume and then added to the blend of oil mix with everything else except the water, which was added slowly at the end to give the required consistency to the emulsion. The enrichment concentration used in the methodology section of this study is the proposed by Funge-Smith (1991), being double of the one recommended by Watanabe et al., (1982).

Appendix II,c

Alkalinity titration method adapted from Stirling (1985) and Clesceri *et al.* (1989).

1) Collect 100 mL sample.
2) Add 5 drops of 4.5 pH indicator.
3) Stir to homogenise colour.
4) Titrate with 0.01 M HCl.
5) Multiply titration values by 5.004 to get mg L^{-1} as $CaCO_3$.

Total hardness titration method adapted from Stirling (1985) and Clesceri *et al.*, (1989).

1) Collect 100 mL sample.
2) Add 1 total hardness indicator tablet.
3) Stir constantly until the tablet is dissolved and the colour is homogeneous.
4) Add 2 mL of Ammonia Buffer Solution. (Ammonia chloride 67.6 g, Ammonia (0.880) 572 mL, Magnesium EDTA 5 g).
5) Titrate with EDTA (Ethylenediaminetetra-acetic acid disodium salt).
6) Take titration values as direct in mg L^{-1} as $CaCO_3$.

Appendix III,a

Proximal analysis of diets used in the experiments (means of three values in percentages).

Diet	Protein	Lipid	Ash	Moisture	Carbohydrate	Crude fibre
Cooked prawns	86.3	3.51	10.89	82		
Cooked mussels	63.33	6.88	10.34	82.07		
Frozen squid	81.74	5.63	6.66	83.74		
Dry pellets*	38.07	13.14	6.70	6.63	31.97	2.55

* Manufactured by Perez Sanchez, N. Institute of Aquaculture, University of Stirling.

Appendix III,b

Histology and Von Kossa's method for calcium identification (taken from the notes of the Histopathology Laboratory, Institute of Aquaculture, University of Stirling).

- Fixation of the samples by bathing them in a 10% neutral buffered formalin (Appendix VI,a) at least 24 hours prior to the embedding process.

- Cassetting, dehydration and impregnation with an embedding paraffin wax in an automatic tissue processor, where the samples go through: 50% Methylated spirit (1 hour) Λ 80% Methylated spirit (2 hours) Λ 100% Methylated spirit (6 hours) Λ 100% Ethanol (4 hours) Λ Chloroform (1 hour) Λ Molten wax (6 hours).

- The sample is blocked out by filling the cassette with liquid wax and then letting it to solidify in a cold plate.

- The sample is then cut (in a microtome), fixed to a clean glass slide and dry in an oven (60 °C.) for at least one hour before staining.

- Staining with Von Kossa method for calcium: Sections to water Λ Wash in several changes of distilled water Λ Place in 2% silver nitrate (aqueous) Λ Expose to bright sunlight or a lamp for 20 to 60 minutes Λ Wash in several changes of distilled water Λ Treat with 2.5% sodium thiosulphate ("Hypo") for 5 minutes Λ Wash well in tap water Λ Counterstain in a light neutral red as desired Λ Dehydrater, clear and mount. (Results: calcium deposits = black; other tissues = red).

Appendix IV,a

Lipid classes determination[2] by HPTLC, Olsen and Henderson (1989).

1) Mark the 2 top corners of 10x10 HPTLC plate, pre-wash in glass tank containing diethyl ether, up to the top of plate.

2) Remove from tank and place in plate holder. Activate in 110 °C oven for approximately 30 minutes.

3) Remove approximately 3 mm silica gel from the top of the plate (this part contains artifacts washed up) with razor blade and turn the plate through 90 degrees. With a soft pencil, lightly mark the position of the origin 1 cm from the bottom and the planned positions of the first (5.5 cm up) and second (9.5 cm up) solvent fronts.

4) Using a 10 µL glass Hamilton syringe, plate each sample on the origin as a 2 mm streak, using 1 to 2 µL of the 10 mg mL^{-1} total lipid stock solution (i.e. using 10 to 20 µg total lipid per 2 mm streak). Plate a known lipid standard for reference. Allow at least 1 cm between each sample streak and between streaks and edge of plate.

[2] Taken from the NERC, Institute of Aquaculture, University of Stirling, methods catalogue (1999).

5) Run plate in first, freshly made, solvent mix up to 5.5 cm mark :

First solvent mix	Small tank	Large tank
Methyl acetate	5 mL	25 mL
Isopropanol	5 mL	25 mL
Chloroform	5 mL	25 mL
Methanol	2 mL	10 mL
0.25% KCl	1.8 mL	9 mL

6) Remove plate from tank and desiccate in desiccator for at least 15 minutes.

7) Run plate in second, freshly made, solvent mix up to 9.5 cm mark :

Second solvent mix	Small tank	Large tank
Hexane	16 mL	80 mL
Diethyl ether	4 mL	20 mL
Glacial acetic acid	0.4 mL	2 mL

8) Remove plate from tank and desiccate for 15 minutes.

9) Spray with copper acetate-phosphoric acid reagent.

10) Char in 160 o C oven for 15 to 20 minutes, as necessary.

11) Remove and cool plate. To store, wrap in tin foil and keep in the dark.

12) Quantify classes by scanning densitometry (Olsen and Henderson, 1989) as soon as possible.

Appendix V,a

Larval Score Index taken from Tayamen and Brown (1999).

No.	CRITERIA TO CHECK	SCORE 0	1	2	sample 1	2	3	4	5	6	7	8	9	10
1.	GUT FULLNESS	gut empty	moderately full (30%-60%)	full gut with faecal strands										
2.	GUT LIPID CONTENT (STATE OF HEPATOPANCREAS)	no lipid vacuoles	very small vacuoles (10%-40%)	relatively full (60%-90%)										
3.	PIGMENTATION (STATE OF CHROMATOPHORES)	no colour/pigments (fully contracted chromatophores)	moderate chromatophores in one area	well dispersed chromatophores (pinkish/amber colouration)										
4.	BODY COLOURATION	gray/dark/bluish on abdominal segment	moderate light orange on abdominal segment	tan/orange/red blend on abdominal segment										
5.	SETATION	disfigured/damaged setae on rostrum, pereopods, telson, uropods	curled/kinked setae on rostrum pereopods, pleopods, telson, uropods	straight/whole setae no deformities on rostrum, pereopods, pleopods, telson, uropods										
6.	MUSCLE TO GUT RATIO	Gut appears wide, muscle narrower on VI abdominal segment	Gut appears narrow and slightly wider muscle	Gut appears narrow and muscle appears thick and wider on VI abdominal segment										
7.	MUSCLE APPEARANCE OF ABDOMEN (APPEARANCE OF ABNORMAL MUSCLES)	opaque/grainy	slightly transparent	clear/transparent smooth										
8.	MELANIZATION (PRESENCE OF BLACK SPOTS)	appendages and parts of body affected	very minor necrosis	no necrosis, absence of black spots										
9.	FOULING ORGANISMS	major parts of body affected	Minor parts of body affected	body clean/absence of protozoans, ciliates,organisms										
10.a	SWIMMING BEHAVIOUR (BETWEEN STAGE VIII TO X)	sluggish/circular motion, erratic movement	moderate movement with head upside down	very active tail first lateral motion/jump-like towards the side										
10.b	PHOTO POSITIVE RESPONSE (BETWEEN STAGE I TO VI)	negative response	slow positive response	fast positive response										
				Total score										
				Larval stage.										

Score ratings 0=poor 1=fair 2=excellent

Number of days ____________ **Batch/Tank No.** ____________________

Date: ____________________ **Scored by:** ____________________

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[1]DA-BFAR-National Freshwater Fisheries Technology Research Centre, Philippines, [2]Institute of Aquaculture, Stirling, UK. UK

(Continuation *Appendix V,a*)

Larval Score Index taken from Tayamen and Brown (1999).

1	GUT FULLNESS	GUT EMPTY OF FOOD **Score 0**	GUT MODERATELY FULL **Score 1**	FULL GUT WITH FAECAL STRANDS **Score 2**
2	GUT LIPID CONTENT (STATE OF HEPATOPANCREAS)	LARVAE APPEARS THIN, NO LIPID GLOBULES VISIBLE **Score 0**	VERY SMALL GLOBULES VISIBLE IN THE DIGESTIVE GLAND **Score 1**	GLOBULES IN THE DIGESTIVE GLAND VISIBLY FULL **Score 2**
3	PIGMENTATION (STATE OF CHROMATOPHORES)	FULLY CONTRACTED CHROMATOPHORES ASSOCIATED W/DARK, BLUISH COLOUR **Score 0**	MODERATE CHROMATOPHORES IN ONE AREA W/LIGHT ORANGE **Score 1**	WELL DISPERSED CHROMATOHORES W/AMBER/TAN/RED PIGMENTS **Score 2**
4	BODY COLOURATION	GREY/DARK/BLUISH APPEARANCE ON ABDOMINAL SEGMENT **Score 0**	MODERATE LIGHT ORANGE APPEARANCE ON ABDOMINAL SEGMENT **Score 1**	TAN/ORANGE/RED/AMBER LIKE APPEARANCE ON ABDOMINAL SEGMENT **Score 2**
5	SETATION	ROSTRUM DAMAGED/ DISFIGURED (Check setae also) **Score 0**	ROSTRUM CURLED BENT/KINKED (Check setae also) **Score 1**	ROSTRUM STRAIGHT/ WHOLE (Check setae also) **Score 2**
6	MUSCLE TO GUT RATIO	GUT APPEARS WIDE, MUSCLE THIN IN VI SEGMENT **Score 0**	GUT APPEARS THIN, MUSCLE IN VI SEGMENT WIDE **Score 1**	GUT APPEARS THIN, MUSCLE IN VI SEGMENT WIDER **Score 2**
7	MUSCLE APPEARANCE OF ABDOMEN (APPEARANCE ABNORMAL MUSCLES)	ABDOMINAL MUSCLE OPAQUE/GRAINY **Score 0**	ABDOMINAL MUSCLE SLIGHTLY CLEAR **Score 1**	ABDOMINAL MUSCLE CLEAR/ TRANSPARENT, SMOOTH **Score 2**
8	MELANIZATION (PRESENCE OF BLACK SPOTS)	BLACK SPOTS SEEN ON APPENDAGES AND/OR BODY **Score 0**	MINOR BLACK SPOTS ON APPENDAGES/BODY **Score 1**	NO BLACK SPOTS (MELANIZATION) **Score 2**
10a	SWIMMING BEHAVIOUR (BETWEEN STAGE VIII TO X)	SLUGGISH MOTION **Score 0**	MODERATE MOVEMENT **Score 1**	FAST, JUMP LIKE MOTION **Score 2**

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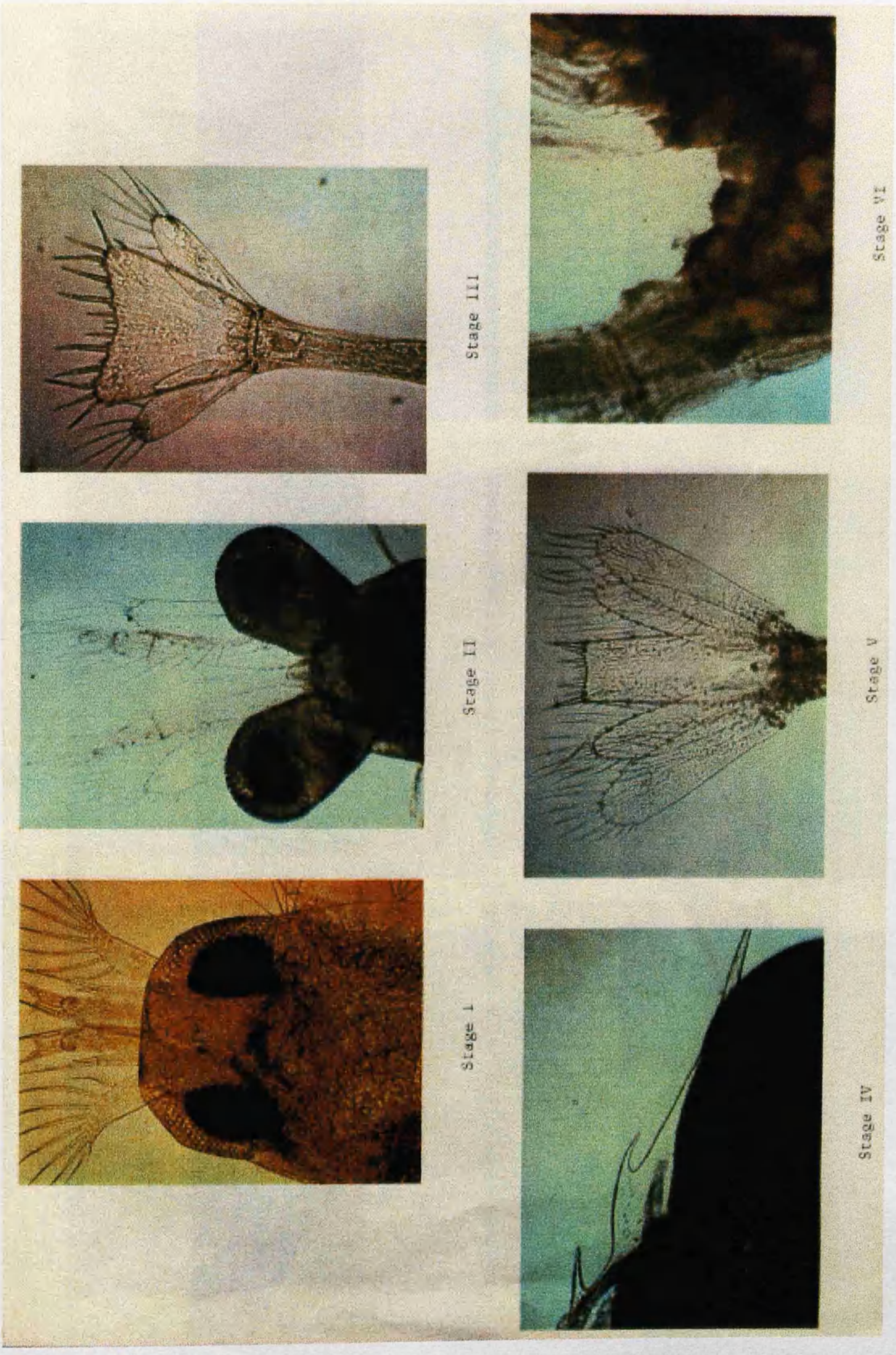
[1]DA-BFAR-National Freshwater Fisheries Technology Research Centre, Philippines, [2]Institute of Aquaculture, Stirling, UK

Appendix V,b

Larval developmental stages according to Uno and Kwon (1968), taken from New and Singholka (1982).



(Continuation *Appendix V,b*)

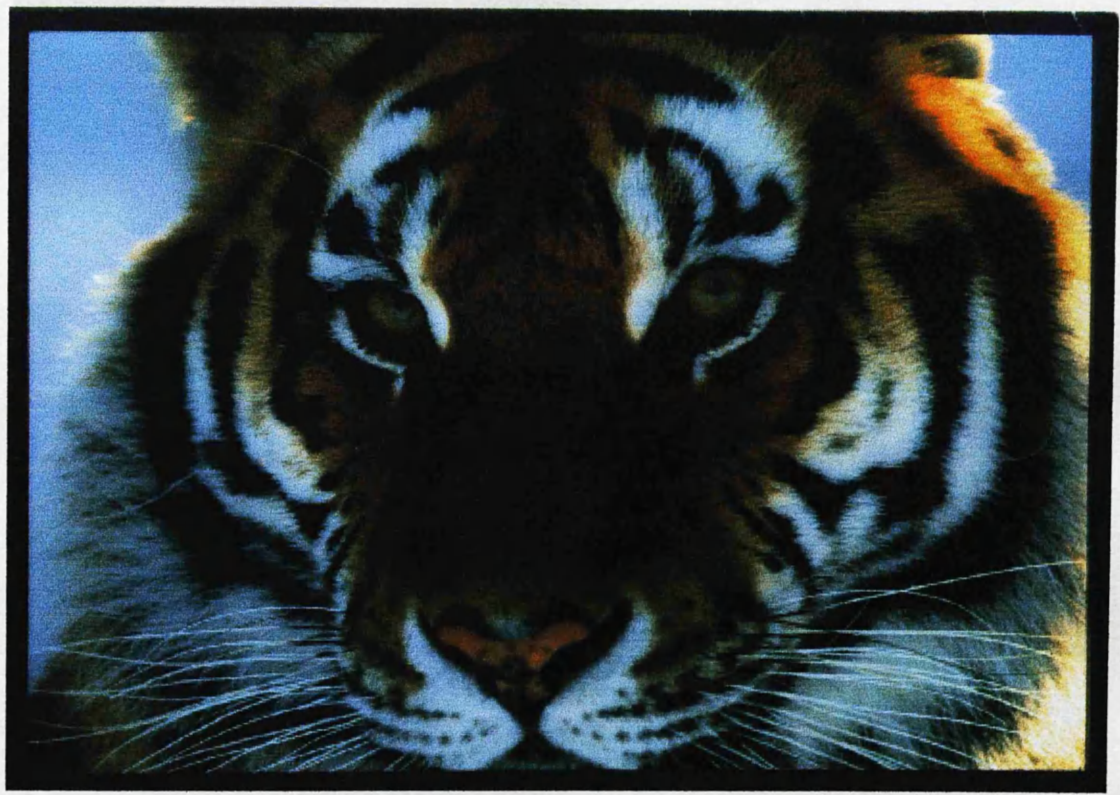
Larval developmental stages according to Uno and Kwon (1969), taken from New and Singholka (1982).

Appendix VI,a

10% Neutral buffered formalin solution

Sodium dihydrogen phosphate (Monohydrated)	4 g
Disodium hydrogen phosphate (anhydrous)	6 g
Formaldehyde	100 mL
Distilled water	900 mL

SMILE



Whenever you see a mountain and you feel the desire to reach the highest point on it....

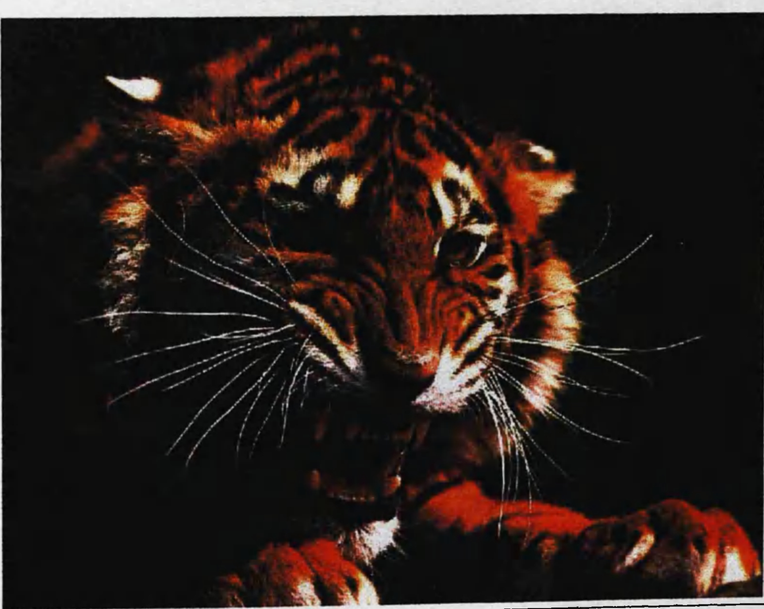
....make sure that your dream is always alive for the rest of your life until the moment that you have satisfied yourself by making it true.

Prepare yourself

and start walking towards that great but unknown ideal, learning from your own path,

but do not forget to enjoy every single moment during your journey.

If something tries to destroy your dream, get ready to fight for it, be clever to avoid wasting precious moments of life and determined to win.



If you have no more choice and finally you have to confront and fight it, then use all your wisdom, courage and strength.

After the battle, take care of your wounds and be proud of yourself for your triumph, but humble and respectful of life, accepting to affront any obstacle that you may find again.

Continue your journey above everything

with the same enthusiasm or more.

Even if there is a river of difficult circumstances in front of you and it is in the way, between you and your desire, analyse them

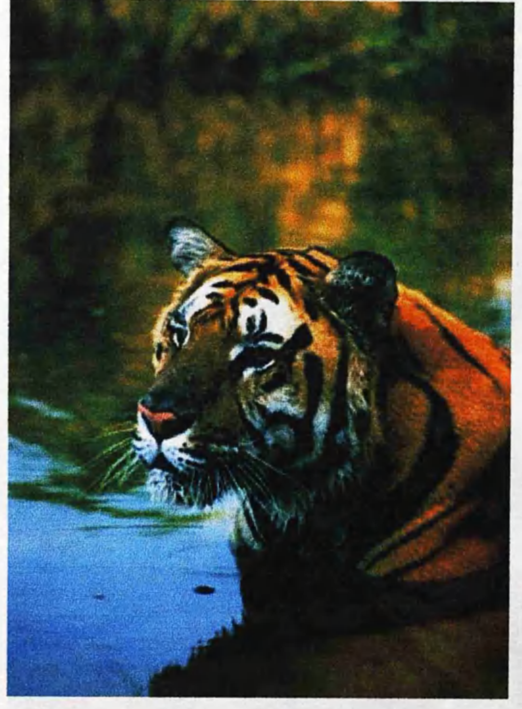
and prepare yourself to enter
the insecure waters.

Charge the uncertainties with
great impetus and jump
into the river.

Swim with your natural instincts, following
your own direction.

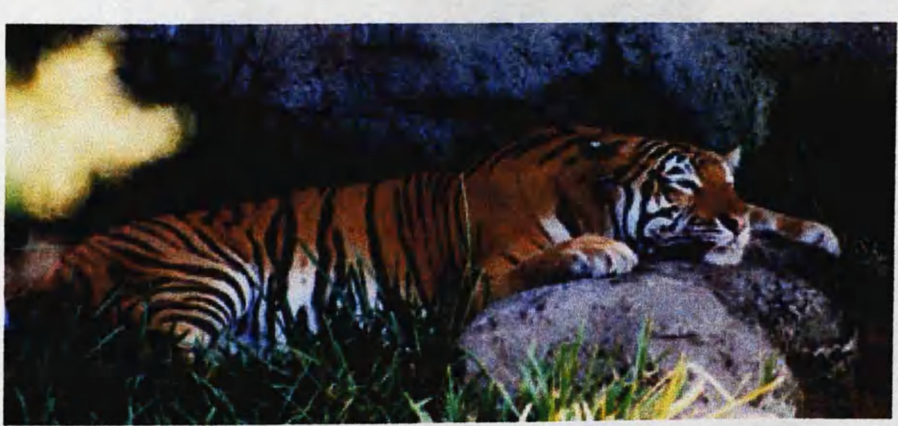
If everything in the river of your life is against you at that moment,
and the current gets stronger, then
swim with more
courage and
determination,

and do not stop until you get to the other side of difficulties.



Get out of that uncertainty and stand on your feet again to continue galloping through your dream.

However, if your body is exhausted by now,



rest for a while,

but not for too long, wake up

and continue until you manage to complete that fascinating adventure in your life.

Because at the end of your journey there will be an instant waiting for you to let you see the past and smile for your achievements.

Afterwards, you will be satisfied and ready to create a new dream on the top of that beautiful mountain.

However, if destiny gives you the gift of meeting your loved one, during your journey, do not be afraid of loosing your dream, of going to the top of the mountain. Your lover will share your passion and desire for going to that mountain, as well as their own desires, with you, so together you can arrive to the top.

Every instant of the journey will be more vivid and you both will be rewarded by sharing all the beautiful moments of it if you mutually

kiss,

comfort,

caress,

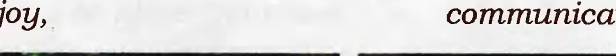
hug,

appreciate,

discus,

think,

protect,



enjoy, *communicate,*

respect, *play,* *enlighten,*

and above all, if you share your dreams

and love each other.

Together you can idealise new illusions, which are the sparks that will motivate you to enjoy your lives. Perhaps together you would like to go to another mountain, the sea or

to give life to your love,

so then you can start another adventure in your lives.

Smile after you have satisfied your desires, remember your path, the adventures and all the emotions that were involved on it, learn from them for your future and try to help other adventurers, but do not forget to laugh about....

difficulties !